bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

1 # Lyophilized cell-free systems display tolerance to organic solvent exposure

2 Marilyn S. Lee[a], Rebecca M. Raig[b,c], Maneesh K. Gupta[b], Matthew W. Lux[a*]

3 [a]US Army Combat Capabilities Development Command Chemical and Biological Center, 8567

4 Ricketts Point Road, Aberdeen Proving Ground, MD 21010 USA, [b]US Air Force Research

5 Laboratory, 2179 12th St., B652/R122 Wright-Patterson Air Force Base, OH 45433 USA, [c]UES

6 Inc., 4401 Dayton-Xenia Rd., Dayton, OH 45432 USA

7

8 [*]Corresponding author:

9 Matthew W. Lux

10 Correspondence: 8567 Ricketts Point Road, Bldg E3549 Room B140, APG, MD USA 21010

11 Phone: 410-436-1448

12 Email: matthew.w.lux.civ@mail.mil

13

14 **Abstract**

15 Cell-free systems offer a powerful way to deliver biochemical activity to the field without cold
16 chain storage. These systems are capable of sensing as well as biosynthesis of useful molecules
17 at the point of need. So far, cell-free protein synthesis (CFPS) reactions have been studied as
18 aqueous solutions in test tubes or absorbed into paper or cloth. Embedding biological
19 functionality into broadly-used materials, such as plastic polymers, represents an attractive
20 goal. Unfortunately, this goal has for the most part remained out of reach, presumably due to
21 the fragility of biological systems outside of aqueous environments. Here, we describe a
22 surprising and useful feature of lyophilized cell-free lysate systems: tolerance to a variety of
23 organic solvents. Screens of individual CFPS reagents and different CFPS methods reveal that
24 solvent tolerance varies by CFPS reagent composition. Tolerance to suspension in organic
25 solvents may facilitate the use of polymers to deliver dry cell-free reactions in the form of
26 coatings or fibers, or allow dosing of analytes or substrates dissolved in non-aqueous solvents,
27 among other processing possibilities.

28

29 **Keywords** Cell-free systems, Protein Stability, Organic Solvents

30

31 ## Introduction

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

32 Cell-free systems are a collection of techniques for activating cellular machinery outside of
33 living cells. Key cell-like functionalities in these systems include cell-free protein synthesis
34 (CFPS)(1–4) and complex metabolism that can both provide energy to the system and produce
35 small molecules of interest (5–9). In these reactions there is no need to maintain cell growth or
36 viability, and removal of the cell membrane facilitates direct addition or measurement of
37 molecules like DNA. These systems have been used for a wide range of applications, including
38 sensing, manufacturing, genetic prototyping, and education (10). There are two main
39 approaches to reconstitute cellular activity *in vitro* (Figure 1A). One approach is the use of crude
40 cell lysates, where cells are broken open and additional building blocks and buffers are added
41 to allow the proteins and other components in the lysate to retain much of their native function
42 (3, 11). Since many undefined components from the cell are still present, there is limited
43 control over biochemical activity. Another approach is the PURE system, where the protein
44 components essential for transcription, translation, and energy cycling are purified before the
45 mixture is reconstituted with energy and substrate solutions (12). This system is much better
46 defined, though it is more expensive to implement and lacks any metabolic enzymes present in
47 lysate that are not individually identified and purified to be included in the reaction.
48
49 A major emerging application of CFPS reactions is rapid field-deployed sensing (13–17) or
50 molecule production at the point of need (17, 18) with minimal required equipment. Though
51 many biological reagents are normally stored frozen or refrigerated, CFPS reactions may be
52 lyophilized for room temperature storage and retain high levels of protein synthesis activity
53 upon rehydration (19–21). CFPS may be used to quickly prototype and implement gene circuit
54 designs that can sense analytes via a variety of mechanisms and produce human readable
55 outputs such as a colorimetric, fluorescent, or even electrochemical reporters (22). Drying CFPS
56 reactions onto paper tickets or cloth has greatly broadened the scope of applications for cell-
57 free techniques (13, 14, 16, 23, 24). Paper-based reactions retain activity, enabling formats
58 similar to pH paper that are less cumbersome for field use. One draw-back to CFPS reactions is
59 that short reaction lifetimes of a few hours make these systems one-use only. A second draw-
60 back is the need to manually add water to activate the lyophilized reaction. Turning to material
61 science to once again redesign the reaction environment could alleviate these issues.
62
63 There is great potential to leverage materials to modulate hydration, and therefore control
64 activation of cell-free systems. Polymers have been widely used to control release timing for
65 cargos that include anything from pharmaceuticals to fertilizers (25, 26). The same principle
66 may be used to extend the lifetime of activity of a detection device. For instance, continual
67 activation of fresh bio-active cargo, such as enzymes, embedded in a material may be achieved
68 by polymer erosion (27, 28). However, this type of formulation requires the biological molecule
69 to be cast into polymers that have low solubility in water, likely necessitating the application of
70 heat or organic solvents. These treatments have a deleterious effect on the activity of most
71 enzymes unless their structure can be protected in some way (29). For a complex mixture like
72 CFPS reactions, it would be reasonable to suspect that such effects on any of the numerous

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

73 enzymes and other constituent components could result in reduction or elimination of overall
74 protein production.
75
76 Non-aqueous enzymology is the study of protein behavior in non-aqueous organic solvents.
77 Over the past few decades, researchers in this field have shown that a subset of purified
78 proteins can, under certain circumstances, maintain their fold and even activity when
79 suspended in organic solvents (30, 31). An important factor influencing protein behavior in
80 organic solvents is the amount of water present in the mixture. Too much water will allow the
81 protein to change conformations, leading to irreversible folding and inactivation. This has been
82 seen in studies titrating organic solvents into aqueous bio-mixtures such as a CFPS reaction (32,
83 33). Therefore, freeze-drying or spray-drying is necessary to carefully remove water from an
84 enzyme sample before solvent addition to better maintain enzyme activity. Indeed, a crystal
85 structure obtained for subtilisin in pure acetonitrile demonstrates that protein structure in this
86 solvent is very similar to the native aqueous structure (31). Tolerance to non-aqueous solvents
87 has been studied for a small subset of purified proteins, but has never before been explored for
88 a complex bio-mixture like a CFPS reaction in which many proteins, nucleic acids, and
89 metabolites must be preserved to maintain transcription and translation activity.
90
91 In this work we describe the effects of exposing lyophilized CFPS reactions to organic solvents.
92 We find that CFPS activity is recovered upon rehydration after exposure of the dried reaction to
93 a variety of organic solvents, though some polar solvents such as ethanol reduce activity.
94 Screens of CFPS reaction recipes, reagent mix components, and solvent removal methods
95 indicate that tolerance to certain solvents is dependent on the presence of additives. Further,
96 we discuss how solvent tolerance of CFPS systems may open the door to new applications by
97 embedding dried CFPS components in new types of polymer materials via solvent casting or
98 sensing analytes in non-aqueous samples.
99

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

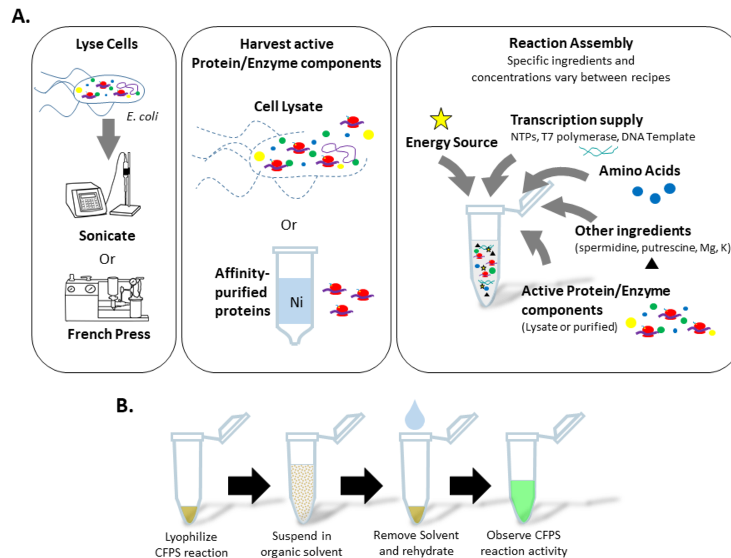



**Figure 1.** (A) Illustration of the composition of a CFPS reaction and variation in published protocols among lysis methods, purity of active enzyme components, and buffer ingredients. (B) Treatment sequence for CFPS reactions includes lyophilization, suspension in an organic solvent, removal of the organic solvent, rehydration, and assessment of CFPS activity via monitoring the appearance of GFP.

## Results and Discussion

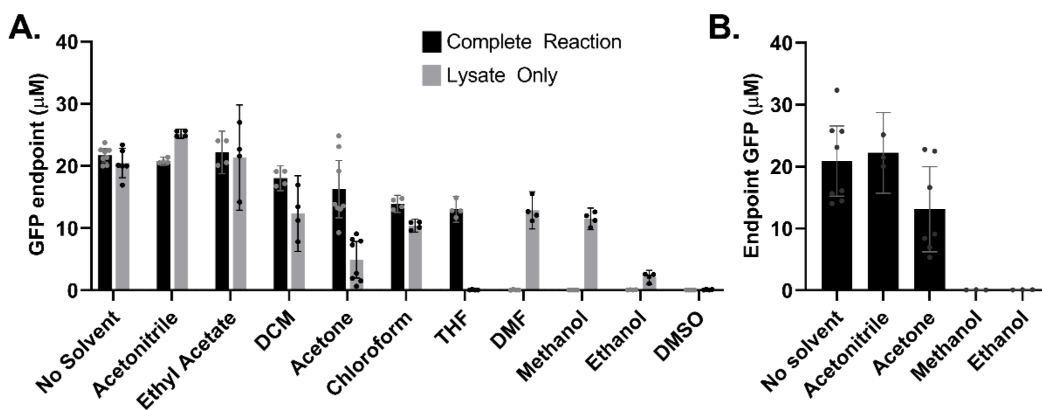
### *Solvent screen*

Initial experiments exposing CFPS reactions to organic solvents utilized an *E. coli* lysate system closely mimicking the recipe constructed by Jewett *et al.* (11) and referred to as PANOx-SP. A panel of solvents including acetone, acetonitrile, chloroform, dichloromethane (DCM), dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethanol, ethyl acetate, methanol, and tetrahydrofuran (THF) were used to challenge both the *E. coli* lysate component lyophilized alone, and the complete lyophilized CFPS reaction mixture. The treatment sequence is summarized in Figure 1B. Solvent properties are summarized in Supplementary Table S1. Each solvent was mixed with the dry cake in microplate wells to form a suspension, then incubated for 1 hour at room temperature. The dried CFPS cake material is largely insoluble in all the organic solvents tested, with insoluble particulates settling quickly after mixing. The solvents were removed by aspiration after brief centrifugation to settle the CFPS material into a pellet. Residual solvent was removed by evaporation at reduced pressure. Then, the reactions were rehydrated with water to reach the desired reaction volume. For samples in which only the lysate is dried and exposed to solvent, the other components of the CFPS reaction were added in the form of aqueous solutions at the rehydration step to initiate protein synthesis activity.

CFPS activity was assessed by tracking GFP production from a plasmid DNA template with a T7 promoter (Figure 2, Supplementary Figure S1). We note here that variability of some

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

127 experimental replicates in this study are higher than in other recent CFPS works that report 6.8-
128 11% variability for individually mixed replicates (34–36). We attribute this difference to either
129 variable loss of some particulate during solvent aspiration or variable penetration of the solvent
130 into particulates during exposures. GFP productivity after organic solvent exposure clearly has
131 some dependence on both the properties of the solvent and the components included during
132 drying and solvent exposure. When the complete reaction is exposed to organic solvents,
133 acetonitrile and ethyl acetate exposure did not result in any loss of activity; acetone, THF,
134 Chloroform, and DCM caused partial loss in activity; and ethanol, DMF, DMSO, and methanol
135 caused total or near total loss of activity. When lysate alone is exposed to solvents, a different
136 pattern emerges: acetonitrile and ethyl acetate again show no loss of activity; acetone, ethanol,
137 chloroform, DMF, methanol, and DCM all yield varying levels of activity; and DMSO and THF
138 completely deactivate the un-supplemented lysate. It is notable that, depending on the solvent
139 type, activity may only be lost for the complete reaction (DMF, Methanol, Ethanol) or only for
140 the lysate (THF), suggesting multiple mechanisms for CFPS inactivation are present.
141
142 There are several possible ways treatment with an organic solvent might impact the
143 productivity of a CFPS reaction. First, the solvent could cause protein unfolding in the lysate or
144 supplemented polymerase and RNase inhibitor protein components in the dry state. Second,
145 the solvent could extract critical resource molecules from the dried cake into solution and
146 cause them to be removed from the system when the solvent is aspirated away. Third, residual
147 solvent not sufficiently removed by evaporation could cause protein unfolding or other
148 inhibition when water is re-introduced (32). Depending on the properties of each solvent, a
149 different combination of these effects may have an impact.
150



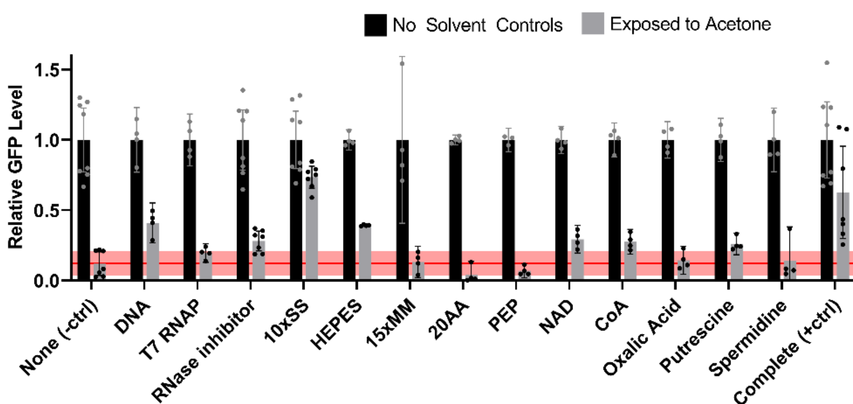

151
152 **Figure 2** (A) Endpoint concentration of GFP in reaction mixtures after lyophilized lysate (grey
153 bars) or complete CFPS reactions (black bars) were exposed to an organic solvent and
154 rehydrated. In the “no solvent” control the dried reactions were rehydrated without solvent
155 exposure. Error bars represent the 95% confidence interval. (B) Activity post-solvent exposure
156 and evaporation at ambient conditions.
157

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

158 We investigated whether specific solvent properties correlated with CFPS productivity results
159 (Supplementary Figures S2 and S3, and Supplementary Tables S2 and S3). Statistically significant
160 correlations were observed for complete reaction exposures but not for lysate alone. These
161 correlations could prove informative for selection of additional solvents. For example, low
162 solvent hydrogen bonding propensity correlated well with higher productivity. Still, due to the
163 complexity of potential solvent interactions with the components of the CFPS reaction,
164 prediction of the compatibility of different solvents or solvent blends is difficult without
165 experiment. Thorough discussion of correlation results may be found in the supplementary
166 information.
167
168 Further experiments were designed to investigate the impact of solvent removal method and
169 lyophilization at larger scale. For some applications, it may not be possible to apply a vacuum to
170 evaporate solvent at reduced pressure. We found that application of a vacuum was
171 unnecessary for maintenance of complete CFPS reaction activity after exposure to acetonitrile
172 or acetone (Figure 2B). Further, washing with volatile acetonitrile did not improve activity after
173 exposure to less-volatile DMSO (Supplementary Figure S4). These experiments indicated that
174 solvent tolerance was not very sensitive to solvent removal method, at least for the subset
175 tested. Applications such as casting dried CFPS into a material may require much larger
176 quantities of lyophilized CFPS powder than the microplate scale used for screening. We
177 lyophilized larger (250 µL vs 15 µL) batches of PANOx-SP CFPS reactions and exposed them to
178 acetone. We found that CFPS activity was maintained (Supplementary Figure S5).
179 Thermogravimetric analysis (TGA) was a useful technique to confirm a water content of less
180 than 3% (wt/wt). Further discussion of these results may be found in the supplementary
181 information.
182
183 *CFPS reaction component screen for acetone tolerance*
184 The observation that the complete dried CFPS reaction performed better than the lysate alone
185 after exposure to acetone, chloroform, or THF suggested that some component of the resource
186 mix might act as a protective additive. To test this hypothesis, components of the resource mix
187 were combined with lysate individually and screened for protective effects upon exposure to
188 acetone and removal of the solvent without a vacuum. In this experiment, three "components"
189 are themselves aqueous mixtures. "10xSS" contains magnesium, potassium, and ammonium
190 glutamate. "15xMM" contains ATP, GTP, UTP, CTP, folinic acid, and tRNA. "20AA" is a mixture of
191 all 20 canonical amino acids. For experimental expediency, samples were rehydrated with a
192 reagent mix containing all reagents, resulting in doubled final concentrations for the individual
193 component screened. Interestingly, only the 15xMM and PEP resulted in statistically significant
194 difference in the productivity due to increased concentration without acetone exposure (Figure
195 S6). Activity decreased in both cases, either because of the change in final concentration or
196 because the component has some negative effect on the lysate during the lyophilization
197 process. After acetone treatment, CFPS ingredients each have varying relative effects on GFP
198 productivity (Figure 3). Plasmid DNA and HEPES buffer both significantly stabilize the lysate.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

199 Remarkably, the 10xSS component alone is sufficient to stabilize the lysate to acetone exposure
200 as well as the complete reaction mixture. This experiment is the first confirmation that altering
201 the composition of the lyophilized CFPS reaction mixture greatly impacts tolerance to organic
202 solvent exposure.
203

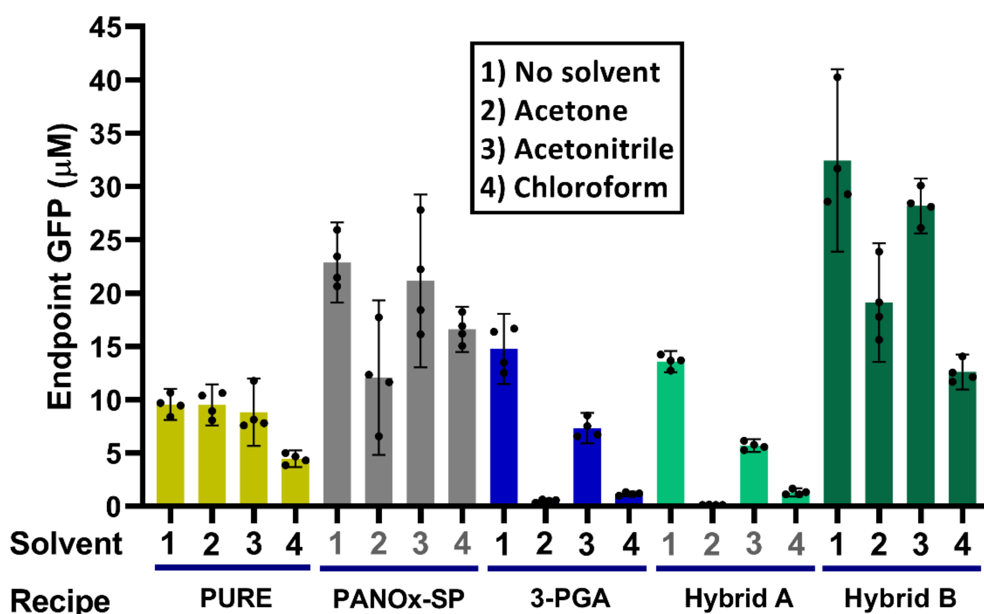



204
205 **Figure 3** Screen of CFPS components for protective effect during acetone exposure. Black bars
206 are results from controls without solvent exposure. Grey bars refer to a 1 hr acetone exposure.
207 Acetone is evaporated under ambient conditions in this experiment. Each set of bars is labeled
208 with the CFPS ingredient used to supplement the lysate sample during drying. The negative
209 control labeled "None" is lysate without any supplemented additive. The positive control
210 labeled "Complete" includes all CFPS ingredients. The red reference line coincides with the
211 mean fraction of GFP productivity of an un-supplemented lysate control exposed to acetone,
212 with lighter red shading representing the 95% confidence interval. GFP levels are normalized to
213 the no solvent control treatment for each component. Figure S6 depicts the data without
214 normalization.
215
216 *Comparing the solvent tolerance of different published CFPS styles*
217 The clear dependence of solvent tolerance on the composition of the reaction led us to
218 investigate solvent tolerance across different published CFPS systems. We tested the
219 performance of three systems: the commercial PURExpress system based on the PURE
220 approach described above (12), the PANOx-SP system used in all experiments above (11), and
221 the 3-PGA system which uses a different strain of *E. coli*, lysis method, and recipe for reaction
222 additives (3, 37). Supplementary Table S4 is a side-by-side comparison of the composition of
223 the CFPS reaction for each recipe. In each case, the reactions are lyophilized and exposed to
224 solvent as described above, with four solvent conditions tested: acetone, acetonitrile,
225 chloroform, or no solvent.
226
227 The GFP productivity results confirm that alternate CFPS recipes respond to solvents in different
228 ways (Figure 4, Supplementary Figure S7). Compared to the lysate-based CFPS reactions, the
229 PURExpress formulation has less starting productivity but also minimal sensitivity to treatments

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

230 with the three solvents tested. Chloroform was the only solvent that significantly reduced GFP
231 productivity in the PURExpress system. On the other hand, the two lysate-based CFPS recipes
232 have very different susceptibility to the organic solvents. 3-PGA is much more susceptible to
233 inactivation by solvent exposure across all three solvent types compared to PANOx-SP.
234
235 To learn what components of the CFPS reaction are responsible for increased solvent
236 sensitivity, the *E. coli* extract and reagent mix components of PANOx-SP and 3-PGA CFPS recipes
237 were swapped so that PANOx-SP extract and 3-PGA reagent mix were combined in the "Hybrid
238 A" mixture and 3-PGA extract and PANOx-SP reagent mix were combined in the "Hybrid B"
239 mixture. These hybrid CFPS recipes were then lyophilized, treated with solvent, and rehydrated
240 as before. The results of this test clearly show that samples containing 3-PGA reagents are more
241 susceptible to solvent inactivation than samples containing PANOx-SP reagents. When
242 combined with PANOx-SP reagent mix, 3-PGA extract performed very similarly to PANOx-SP,
243 indicating that differences in the cell extract were not the primary factor contributing to solvent
244 sensitivity. While our focus here was on differences in solvent tolerance and not relative
245 productivity of each system, the hybrid mixtures offer an interesting opportunity to interpret
246 productivity trends in the no solvent cases. It is first worth noting that our yields here are lower
247 than those in the literature for each case, which could be a result of the lyophilization step,
248 unintentional differences in protocol execution, DNA construct, reaction conditions, etc.
249 Nonetheless, we observed that despite no significant difference for the base PANOx-SP and 3-
250 PGA systems, we saw a significant decrease from PANOx-SP to Hybrid A, and a significant
251 increase from 3-PGA to Hybrid B. This result indicates that, at least for our specific
252 implementation and conditions, the PANOx-SP reagent mix yields more protein than 3-PGA
253 reagent mix. Among many differences in the supplemented reagents, a major difference is the
254 type of energy source. PANOx-SP makes used of phosphoenolpyruvate (PEP) to regenerate ATP
255 in the reaction, while 3-PGA utilizes maltodextrin to energize the reaction through substrate
256 phosphorylation and glycolysis. Further experimentation is needed to elucidate specific
257 components leading to solvent sensitivity.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.




258
259 **Figure 4** Comparison of different CFPS recipes challenged with organic solvents. Endpoint GFP
260 concentrations are compared for five CFPS recipes after exposure to organic solvents. Error
261 bars represent the 95% confidence interval. Recipe labeled "Hybrid A" is a CFPS reaction mixing
262 PANOx-SP *E. coli* extract and 3-PGA reagent mix. "Hybrid B" is a mixture of 3-PGA extract and
263 PANOx-SP reagent mix.
264
265 *Summary*
266 The study of the interaction between cell-free reaction components and organic solvents
267 revealed several new findings. Lyophilized CFPS reactions tolerate exposure to a variety of
268 organic solvents without loss or with only partial loss of transcription and translation activity.
269 The degree to which activity is lost by solvent exposure depends on the type of solvent and the
270 ingredients in the lyophilized mixture. Experiments comparing different CFPS recipes indicate
271 that differences in the cell extract preparation are less important for solvent tolerance than the
272 composition of the other ingredients in a CFPS reaction. For acetone exposure, 10xSS alone was
273 sufficient to provide solvent tolerance to the *E. coli* lysate, and DNA and HEPES buffer
274 components provided partial protection.
275
276 These newly-discovered characteristics of cell-free systems open the door to many previously
277 elusive applications and directions of study. For instance, the ability to process the biological
278 components of cell-free systems in organic solvents without loss of activity is likely to enable
279 casting these systems into polymeric matrices such as polyurethane that do not dissolve in
280 water. It may also be possible to dose a cell-free sensor system with non-aqueous substrates or
281 analytes. Further study of cell-free systems' interactions with organic solvent could lead to
282 fundamental insights in non-aqueous enzymology and protein folding. The tolerance of CFPS to
283 solvent when lyophilized raises the question of whether complex systems like the ribosome can

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

284 maintain activity in an organic solvent either naturally or through engineering. The ability to
285 study these processes in a non-native context is an advantage unique to cell-free systems.
286

287 **Materials and Methods**

288 *Reagents*

289 The vendor and catalog number for each organic solvent used in this study is provided in
290 Supplementary Table S1 describing solvent properties. Unless otherwise noted, all other
291 reagents were purchased from Millipore Sigma, St. Louis, MO. Commercial PURExpress kits
292 were purchased from New England Biolabs, Ipswich MA. The plasmid template for expression
293 of GFP via CFPS is PY71sfGFP with genbank accession number MT346027 (38). Plasmid DNA was
294 purified from transformed *E. coli* using a Promega PureYield plasmid midiprep kit, followed by
295 ethanol precipitation to further concentrate and purify the DNA. DNA is stored in RNase and
296 DNase-free water at -20°C until reaction assembly.
297

298 *CFPS reaction preparation*

299 PANOx-SP cell extract is prepared from shake flask cultures of *E. coli* BL21 Star (DE3) according
300 to the growth and sonication protocol detailed previously by Kwon and Jewett (11). 3-PGA cell
301 extract is prepared from shake flask cultures of *E. coli* BL21 Rosetta2 and lysed by pressure with
302 a Microfluidizer cell homogenizer. The growth and lysis methods were based closely on
303 protocols published previously by Noireaux et al (3, 37).
304

305 Lysates were aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until use in CFPS
306 reaction assembly. To assemble reactions, CFPS components including PURExpress kit solutions,
307 cell lysates, and reagent stock solutions, were thawed on ice, then combined with DNAse free
308 water to reach the final concentrations listed in Supplementary Table S4. Reaction mixtures
309 were well mixed and distributed 15 µL per well into 96 well, v-bottom, polypropylene plates
310 (Costar 3357). Further details on the preparation of CFPS lysates and reagent mixes for PANOx-
311 SP and 3-PGA recipes, as well as lyophilization methods are described in the supplemental
312 information.
313

314 *Solvent treatment and rehydration of CFPS reactions*

315 All solvent treatments are performed in a chemical fume hood. 100 µL of each solvent was
316 added to the lyophilized CFPS reaction mixture in designated wells. No solvent was added to
317 lyophilized control reactions. Wells were sealed with a flexible polypropylene mat (Costar 3080)
318 to prevent evaporation. Reactions were incubated with solvent for one hour at room
319 temperature. Following solvent incubation, plates were briefly spun at low speed in a
320 centrifuge to settle the insoluble CFPS reaction components, then solvents were removed by
321 aspiration with a pipette without disturbing the pellet. The residual solvent was removed by
322 evaporation. This was achieved either by allowing the plate to sit uncovered in the fume hood
323 for ambient evaporation, or applying a vacuum at room temperature using a vacuum oven with
324 the heating element turned off for 20 minutes. After solvent removal, all reactions were

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

325 rehydrated with 15 µL of DNase free water to return CFPS reaction components to their original
326 aqueous concentration. Reaction assembly and solvent treatment methods for the PANOx-SP
327 CFPS component screen are described in the supplemental information.
328
329 *Monitoring GFP formation via microplate reader*
330 Immediately after rehydration, plates were sealed with a polypropylene mat, transferred to a
331 BioTek Synergy H1 microplate reader, and incubated at 30°C for 8 hours. Formation of GFP
332 fluorescence e was monitored with ex/em: 485/528 nm with a gain of 100. GFP readings in RFU
333 were converted to µM GFP using fluorescence measurements of purified GFP standards.
334
335 **Abbreviations**
336 CFPS = cell-free protein synthesis
337 GFP = green fluorescent protein
338 DCM = dichloromethane
339 DMF = dimethylformamide
340 DMSO = dimethylsulfoxide
341 THF = tetrahydrofuran
342
343 **Acknowledgements**
344 We thank Stephanie Cole for providing materials for the 3-PGA experiments. We also thank
345 Michael Jewett's laboratory at Northwestern University and Vincent Noireaux's laboratory at
346 University of Minnesota for sharing advice and detailed protocols. This work was made possible
347 by funding from the Office of the Secretary of Defense's Applied Research for the Advancement
348 of Science and Technology Priorities program. Follow-on funding was provided by the CCDC CBC
349 Surface Science Initiative. This work was completed while author Marilyn Lee held an NRC
350 research associateship supported by the CCDC CBC Biological Engineering for Applied Materials
351 Solutions (BEAMS) program.
352
353 **Supporting Information**
354 Additional methods details; kinetics data; data and additional interpretation for solvent
355 property correlations, removal of residual solvent by washing, and increased lyophilization
356 volumes
357
358 **References**
359
360 1. M. C. Jewett, J. R. Swartz, Mimicking theEscherichia coli cytoplasmic environment
361 activates long-lived and efficient cell-free protein synthesis. *Biotechnology and*
362 *Bioengineering* **86**, 19–26 (2004).

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.


363 2. M. C. Jewett, K. A. Calhoun, A. Voloshin, J. J. Wuu, J. R. Swartz, An integrated cell-free
364 metabolic platform for protein production and synthetic biology. *Molecular Systems*
365 *Biology* **4**, 220 (2008).

366 3. Z. Z. Sun, *et al.*, Protocols for Implementing an Escherichia coli Based TX-TL Cell-Free
367 Expression System for Synthetic Biology. *Journal of Visualized Experiments* (2013)
368 https:/doi.org/10.3791/50762 (December 31, 2019).

369 4. F. Caschera, V. Noireaux, Synthesis of 2.3 mg/ml of protein with an all Escherichia coli cell-
370 free transcription–translation system. *Biochimie* **99**, 162–168 (2014).

371 5. Y. Y. Wu, *et al.*, System-level studies of a cell-free transcription-translation platform for
372 metabolic engineering. *bioRxiv* (2017) https:/doi.org/10.1101/172007 (December 31,
373 2019).

374 6. D.-M. Kim, J. R. Swartz, Regeneration of adenosine triphosphate from glycolytic
375 intermediates for cell-free protein synthesis. *Biotechnology and Bioengineering* **74**, 309–
376 316 (2001).

377 7. Q. M. Dudley, A. S. Karim, M. C. Jewett, Cell-free metabolic engineering: Biomanufacturing
378 beyond the cell. *Biotechnology Journal* **10**, 69–82 (2015).

379 8. A. S. Karim, M. C. Jewett, A cell-free framework for rapid biosynthetic pathway
380 prototyping and enzyme discovery. *Metabolic Engineering* **36**, 116–126 (2016).

381 9. A. S. Karim, J. T. Heggestad, S. A. Crowe, M. C. Jewett, Controlling cell-free metabolism
382 through physiochemical perturbations. *Metabolic Engineering* **45**, 86–94 (2018).

383 10. A. D. Silverman, A. S. Karim, M. C. Jewett, Cell-free gene expression: an expanded
384 repertoire of applications. *Nature Reviews Genetics* **21**, 151–170 (2020).

385 11. Y.-C. Kwon, M. C. Jewett, High-throughput preparation methods of crude extract for
386 robust cell-free protein synthesis. *Scientific Reports* **5** (2015).

387 12. Y. Shimizu, *et al.*, Cell-free translation reconstituted with purified components. *Nature*
388 *Biotechnology* **19**, 751–755 (2001).

389 13. A. Gräwe, *et al.*, A paper-based, cell-free biosensor system for the detection of heavy
390 metals and date rape drugs. *PLOS ONE* **14**, e0210940 (2019).

391 14. M. K. Takahashi, *et al.*, A low-cost paper-based synthetic biology platform for analyzing gut
392 microbiota and host biomarkers. *Nature Communications* **9** (2018).

393 15. M. P. McNerney, *et al.*, Point-of-care biomarker quantification enabled by sample-specific
394 calibration. *Science Advances* **5**, eaax4473 (2019).

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

395 16. A. Meyer, *et al.*, Organism Engineering for the Bioproduction of the
396 Triaminotrinitrobenzene (TATB) Precursor Phloroglucinol (PG). *ACS Synthetic Biology* **8**,
397 2746–2755 (2019).

398 17. K. Pardee, Perspective: Solidifying the impact of cell-free synthetic biology through
399 lyophilization. *Biochemical Engineering Journal* **138**, 91–97 (2018).

400 18. B. C. Bundy, *et al.*, Cell-free biomanufacturing. *Current Opinion in Chemical Engineering* **22**,
401 177–183 (2018).

402 19. D. K. Karig, S. Bessling, P. Thielen, S. Zhang, J. Wolfe, Preservation of protein expression
403 systems at elevated temperatures for portable therapeutic production. *Journal of The*
404 *Royal Society Interface* **14**, 20161039 (2017).

405 20. N. E. Gregorio, *et al.*, Unlocking Applications of Cell-Free Biotechnology through Enhanced
406 Shelf Life and Productivity of *E. coli* Extracts. *ACS Synthetic Biology* (2020)
407 https:/doi.org/10.1021/acssynbio.9b00433 (April 2, 2020).

408 21. M. T. Smith, S. D. Berkheimer, Lyophilized *Escherichia coli* -based cell-free systems for
409 robust, high-density, long-term storage. *BioTechniques* **56** (2014).

410 22. P. Sadat Mousavi, *et al.*, A multiplexed, electrochemical interface for gene-circuit-based
411 sensors. *Nature Chemistry* **12**, 48–55 (2020).

412 23. K. Pardee, *et al.*, Paper-Based Synthetic Gene Networks. *Cell* **159**, 940–954 (2014).

413 24. T. T. M. Duyen, *et al.*, Paper-based colorimetric biosensor for antibiotics inhibiting
414 bacterial protein synthesis. *Journal of Bioscience and Bioengineering* **123**, 96–100 (2017).

415 25. M. W. Tibbitt, J. E. Dahlman, R. Langer, Emerging Frontiers in Drug Delivery. *Journal of the*
416 *American Chemical Society* **138**, 704–717 (2016).

417 26. H. Tian, *et al.*, Biobased Polyurethane, Epoxy Resin, and Polyolefin Wax Composite Coating
418 for Controlled-Release Fertilizer. *ACS Applied Materials & Interfaces* **11**, 5380–5392 (2019).

419 27. D. Ding, Q. Zhu, Recent advances of PLGA micro/nanoparticles for the delivery of
420 biomacromolecular therapeutics. *Materials Science and Engineering: C* **92**, 1041–1060
421 (2018).

422 28. N. Kamaly, B. Yameen, J. Wu, O. C. Farokhzad, Degradable Controlled-Release Polymers
423 and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release. *Chemical Reviews*
424 **116**, 2602–2663 (2016).

425 29. P. B. Dennis, A. Y. Walker, M. B. Dickerson, D. L. Kaplan, R. R. Naik, Stabilization of
426 Organophosphorus Hydrolase by Entrapment in Silk Fibroin: Formation of a Robust

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

427 Enzymatic Material Suitable for Surface Coatings. *Biomacromolecules* **13**, 2037–2045
428 (2012).

429 30. A. M. Klibanov, Improving enzymes by using them in organic solvents. *Nature* **409**, 241–
430 246 (2001).

431 31. J. L. Schmitke, L. J. Stern, A. M. Klibanov, Comparison of x-ray crystal structures of an acyl-
432 enzyme intermediate of subtilisin Carlsberg formed in anhydrous acetonitrile and in water.
433 *Proceedings of the National Academy of Sciences* **95**, 12918–12923 (1998).

434 32. J. E. Kay, M. C. Jewett, A cell-free system for production of 2,3-butanediol is robust to
435 growth-toxic compounds. *Metabolic Engineering Communications* **10**, e00114 (2020).

436 33. N. Doukyu, H. Ogino, Organic solvent-tolerant enzymes. *Biochemical Engineering Journal*
437 **48**, 270–282 (2010).

438 34. S. D. Cole, *et al.*, Quantification of Interlaboratory Cell-Free Protein Synthesis Variability.
439 *ACS Synthetic Biology* **8**, 2080–2091 (2019).

440 35. J. L. Dopp, Y. R. Jo, N. F. Reuel, Methods to reduce variability in E. Coli-based cell-free
441 protein expression experiments. *Synthetic and Systems Biotechnology* **4**, 204–211 (2019).

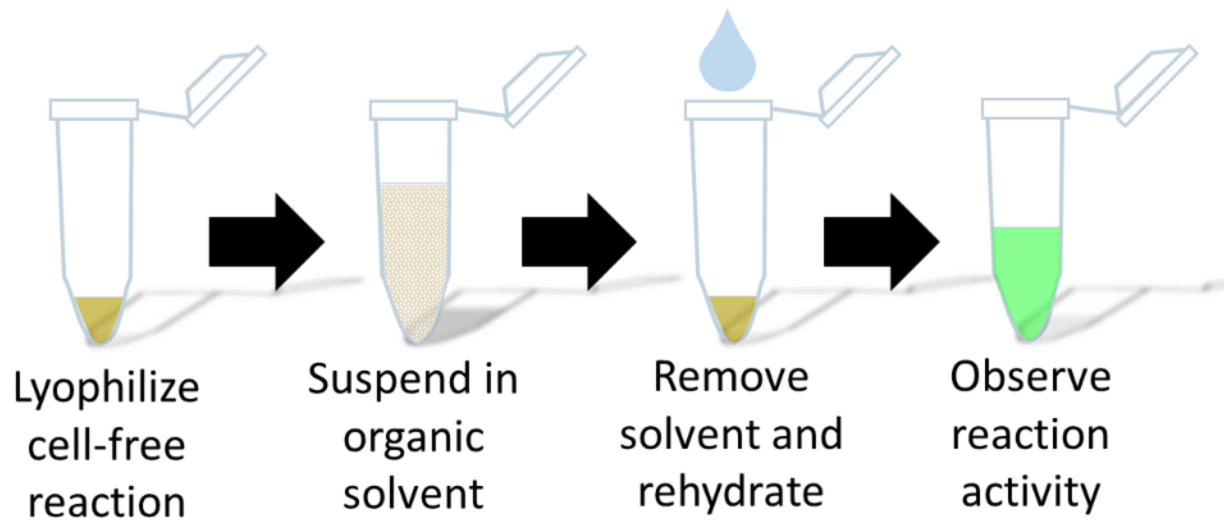
442 36. A. D. Silverman, N. Kelley-Loughnane, J. B. Lucks, M. C. Jewett, Deconstructing Cell-Free
443 Extract Preparation for *in Vitro* Activation of Transcriptional Genetic Circuitry. *ACS*
444 *Synthetic Biology* **8**, 403–414 (2019).

445 37. J. Garamella, R. Marshall, M. Rustad, V. Noireaux, The All *E. coli* TX-TL Toolbox 2.0: A
446 Platform for Cell-Free Synthetic Biology. *ACS Synthetic Biology* **5**, 344–355 (2016).

447 38. B. C. Bundy, J. R. Swartz, Site-Specific Incorporation of *p* -Propargyloxyphenylalanine in a
448 Cell-Free Environment for Direct Protein−Protein Click Conjugation. *Bioconjugate*
449 *Chemistry* **21**, 255–263 (2010).

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.11.121418; this version posted June 12, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

450 **For Table of Contents Only**




451