Constructing cell-free expression systems for low-cost access

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ABSTRACT

Cell-free systems for gene expression have gained attention as platforms for the facile study of genetic circuits and as highly effective tools for teaching. Despite recent progress, the technology remains inaccessible for many in low and middle income countries due to the expensive reagents required for its manufacturing, as well as specialized equipment required for distribution and storage. To address these challenges, we deconstructed processes required for cell-free mixture preparation and developed a set of alternative low-cost strategies for easy production and sharing of extracts. First, we explored the stability of cell-free reactions dried through a low-cost device based on silica beads, as an alternative to commercial automated freeze dryers. Second, we report the positive effect of lactose as an additive for increasing protein synthesis in maltodextrin-based cell-free using either circular or linear DNA templates. The modifications were used to produce active amounts of two high-value reagents: the isothermal polymerase *Bst* and the restriction enzyme BsaI. Third, we demonstrated the endogenous regeneration of nucleoside triphosphates and synthesis of pyruvate in CFS based on PEP and MDX. We exploited this novel finding to demonstrate use of a cell-free mixture completely free of any exogenous NTPs to generate high yields of sfGFP expression. Together, these modifications can produce desiccated extracts that are 203-424-fold cheaper than commercial versions. These improvements will facilitate wider use of CFS for research and education purposes.

KEYWORDS: Cell-free protein synthesis, lyophilisation, NTPs, lactose, low-cost, maltodextrin

INTRODUCTION

Since discovery of the functional relationship between mRNA and protein by Nirenberg and Mattaei in the early 1960s [1] , cell-free systems (CFS) have become powerful tools with broad applications now in synthetic biology, ranging from the study of artificial gene circuits and synthetic cells to protein production [2-4] . The technology relies on *in vitro* transcription-translation systems which employ cell-extracts as source of ribosomes and auxiliary transcriptional factors [5], or reconstitution of purified cell components in the case of the PURE system[6, 7]. In addition to transcription-translation machinery, CFS requires an adequate supply of key elements such as amino acids, crowding reagents, salts, nucleotide triphosphate (NTPs), homeostatic environment, and an ATP regeneration system [8]. There have been numerous reports of improvements in the way extracts are generated and fed. Several approaches have been used to find better balance between protein yields and reagent cost, where nucleoside triphosphate and energy source represent more than fifty per cent of the total cost of reactions [9]. Energy substrates generally contain high-energy phosphate bonds, generating the ATP, necessary to carry out protein synthesis and other metabolic processes[10] through simple phosphorylation reactions. However, phosphate donors, such as phosphoenol pyruvate (PEP), creatine phosphate (CP), 3-phosphoglycerate (3-PGA) and fructose 1,6-bisphosphate are relatively expensive and require cold-chain during storage and distribution, limiting the adoption of these cell-free technologies in low-resource settings, or at larger scale [11] . To address this issue, ATP regeneration systems based on multi-enzyme reaction cascades associated with glycolysis and oxidative phosphorylation metabolism have been implemented [12-14]. An example of this kind of energy source is maltodextrin (MDX), which also enhances protein production by limiting the production of excess phosphate levels in the reaction [15]. Due to its low-cost and high efficiency, the maltodextrin system was adopted and improved by Caschera et al. (2015), coupled with hexametaphosphate (HMP), as a phosphate donor to stimulate glycolysis [16]. However, this ATP regeneration system has not been widely adopted as an energy source in CFS despite it has proven its efficiency for characterizing toehold sensors in low-cost contexts [17].

So far, most efforts to reduce the costs of production and implementation of CFS have focused on alternative ATP regeneration systems, choice of reagents and the optimisation of working concentrations [11, 18, 19], leaving aside other critical aspects. For example, CFS have gained attention in diagnostics due to its capacity to be lyophilised as pellets or in paper matrices, permitting the expression of a synthetic gene network in a point of care assay under contained conditions[20, 21]. Applications include the detection of Zika virus by coupling cell-free technology with isothermal RNA amplification and a toehold switchm[22] and the measurement of water contaminants through the ROSALIND (RNA Output Sensors Activated by Ligand Induction) system[23]. These advances have enabled the fabrication of low-cost, rapid diagnostics, but some remaining steps, such as the lyophilisation of cell-free reaction components that are required for storage and distribution, still rely on access to expensive equipment.

To improve access to this technology, we have developed three novel approaches to reducing cost. First, we evaluated the capacity of silica beads coupled with a low vacuum to dry cell-free components based on two independent energy sources: PEP and MDX, using sugars as lyoprotectant agents to stabilise the mixtures. This is the first demonstration of the use of maltodextrin in a lyophilized mixture. These conditions allowed us to maintain 45 % and 75 % of protein synthesis capacity after two weeks of storage at room temperature in mixtures based on PEP and MDX, respectively, compared to fresh preparations.

Second, we found that the addition of lactose enhanced cell-free reactions, seeing a 188 % increase in protein yield when maltodextrin was used as an energy source, giving comparable yields to those obtained with PEP. On this basis, we developed a modified version of the MDX cell-free formulation used it to produce active protein reagents: *Bst* DNA polymerase (commonly used in LAMP assays)

using plasmid as template, and the Type IIS restriction enzyme BsaI using linear DNA as template. We developed a technique for efficient use of linear DNA templates in the absence of stabilisers such as GamS or Chi DNA [24, 25].

Third, we found that endogenous biosynthesis of nucleoside mono and triphosphate remains active in CFS reactions based on PEP. Thus, reactions could be composed without the addition of NTPs or NMPs[11] in the mixture, contrary to normal practice. This allowed us to develop an ultra-low-cost cell-free system capable of producing 16.9 µM sfGFP using just cell-extract, PEP, amino acids, salts and lactose. This is likely to be the forerunner of a new class of cell-free expression systems that further employ closed biochemical circuits to regenerate essential reactants, and to lower costs. The combined improvements described in this work facilitate the remote production of useful protein reagents and reduce the dependence on expensive equipment and supplies.

RESULTS AND DISCUSSION

Drying cell-free reactants using a low-cost protocol with sugar protectants

In recent years, cell-free systems have gained attention in the diagnostics field due to their versatility over traditional methods, particularly in high-throughput approaches using small volume reactions and distribution to the point of care in a lyophilised form[20, 23, 26]. However, the use of high-cost equipment required to lyophilise cell-free extracts remains a limitation for local production of these diagnostic reactions. To overcome this challenge, we tested the use of a low-cost alternative (Fig 1A) to high-tech freeze dryers (Fig 1B) in two cell-free formulations based on different energy regeneration systems (Tables S1-S4). We observed that when samples using PEP as energy source were frozen and lyophilised without the addition of lyoprotectant in a computer-controlled commercial device (Table S4), they did not show a reduction in protein production one day after lyophilisation (Fig 1C, top panel). However, only 31 % of the initial activity remained after two weeks of storage at room temperature (23°C). We also tested a low-cost drying device, where samples were simply kept overnight under low vacuum in a desiccator containing dry silica gel. When this device was used, the recovery was around 30 % after 1 day, and 20 % after 2 weeks for the PEP energised reactions (Fig 1C, top panel). Despite this drop in the recovery, the samples still showed high absolute levels of protein synthesis activity, since yields for fresh samples were in the range of 40 µM of sfGFP. Longer term storage of all dried samples was maintained in light-shielded, vacuum-bagged containers with desiccant in an argon-flushed, anoxic environment.

In contrast, cell-free reactions having maltodextrin as an energy source, showed a better recovery after 2 weeks (50 %) when simply dried over silica, compared to when the commercial freeze dryer was used (20 %). Interestingly, the silica-dried samples showed the same level of stability from day 1 until two weeks later after storage of the dry reactions at room temperature (Fig 1C, bottom panel). This level of protection may be due to the presence of maltodextrin and PEG 8000[27, 28]in the reaction mixes. However, this does not explain the lower stability of samples dried by lyophilisation. A possible explanation for this effect is the formation of ice crystals during the slow-freezing step before the lyophilisation, which can cause structural damage in the cellular constituents present in the reaction mixture[29]. Given that the PEP and MDX formulations (Tables S3-S4) did not show a consistent difference in stability after drying or lyophilisation, we evaluated the stabilising and lyoprotectant properties of different sugars. Sugars are thought to act as water substitutes against dehydration through hydrogen bond interactions with dehydrated proteins[30-33], contributing to stabilisation of preferred protein conformations. Five sugars (trehalose, maltose, lactose, sucrose and raffinose) at range concentration from 0 to 120 mM (Table S5) were tested as protectants and compared to fresh cell-free reactions, evaluating the percentage of recovered activity at one day and two-weeks after

lyophilisation (Fig 2A-J,Fig S2A-J, duplicate reactions are described in Fig S1). It has been previously shown that trehalose can be an effective lyoprotectant in cell-extracts[34, 35]. However, for PEP containing extracts, we observed that sucrose and raffinose were the most effective stabilizers in both drying systems, showing activities of 75 % and 45 % after lyophilisation or silica-drying, respectively (Fig S2B and S2E). These yields were obtained adjusting the sugar concentration in the reaction mixtures, reaching a maximum level of protection at 120 mM (Fig S2). In contrast, the rest of the sugars (maltose, lactose, trehalose) exhibited a negative effect as the concentration increased (Fig S2). In part, this may be due to molar ratios of stabilizer and protein[36]. In the case of trehalose, previous studies suggested that a high concentration of this disaccharide can inhibit protein expression due to its high affinity for water molecules[35, 37], and consequent displacement of water from biomolecules. This might explain the low percentage of activity observed in samples dosed with this sugar (Fig S2A and S2F). Positive effects were seen when sugars were added as protectants along with maltodextrin in both drying processes, more pronounced when using the low-cost silica drying device. The best results (around 75 % of the original activity) were obtained when 5 mM trehalose, maltose or lactose were added to the reaction mix (Fig 2F,H-I;Table S3) , while the optimal concentration for sucrose was 5-15 mM (Fig 2G). The only two conditions that showed a high level of stabilisation after lyophilisation were addition of maltose at low concentrations (5 and 15 mM) and sucrose at all the tested concentrations (Fig 2 G-H and Fig S2G). Using trehalose, sucrose, maltose (Fig 2F-H), the protein yields were the same after one day and two weeks of dry storage. On the basis of these observations, we decided to add sucrose as a protectant in reaction mixtures based on either PEP (120 mM, Table S4) or maltodextrin (15 mM, Table S3), for both lyophilisation and silica-drying procedures due to the positive effect shown by this sugar under a wide range of conditions. This allowed us to use the cheapest sugar (USD $0.0066 per gram of sucrose) to minimise cost. Next, we sought to demonstrate the effectiveness of the approach by sending dried samples based on MDX as energy source and protected with 15 mM sucrose for testing in Mexico and Chile (Fig S3A-B). The reaction mixtures were stable two weeks after drying, including transatlantic shipping and delays due to customs services in each country. In addition, the same batch of reactants was also successfully evaluated after 3 months (Fig S3C) in the UK along with the protective effect of sucrose at different concentrations in the MDX formulation (Fig S3D, Tables S3 and S5), showing up to % 60 recovery compared to fresh samples and demonstrating the robustness of our system in terms of stability and cost (Fig S8C). In summary, silica-based drying provides a new low-cost alternative to lyophilisation for drying cell-free reactions with potential use in diagnostic and education, using a cheap energy source of energy (MDX) and sucrose as protective agent, which allows world-wide shipping and storage of reactants at room temperature.

Lactose enhances cell-free reactions yields

We observed that some sugars stimulated protein production in our cell-free expression systems, and we decided to evaluate their properties more systematically as additives. Sugars have been used as secondary energy sources in cell-free reactions to enhance protein yields[9, 10, 38-40]. Recently, Moore et al.[41] demonstrated that combined use of 3-phosphoglyceric acid (3-PGA), with glucose-6-phosphate (G6P) as secondary energy source, improved protein production in *Streptomyces* cell-free reactions 6-fold. Similarly, a beneficial effect on protein yields was observed when E. coli cell-extracts were supplemented with 30 mM d-ribose in a system based on MDX [42]. Further, lyophilized *E. coli* cell-free extracts stored at -80°C and rehydrated after two weeks showed increased activity if prepared with maltose, trehalose or lactose, with PEP as main energy force, suggesting that sugars were useful additives in cell-free reactions[34]. These observations were consistent with our results with fresh samples, as an enhancement in protein production was seen, when PEP or MDX was used as main source energy and the relevant sugars were added (Fig 2, Table S5-S6). Samples supplemented with trehalose recorded a maximum peak of expression (125 %) at 3.7 mM concentration (Table S5), while in cell-free reactions containing maltose and lactose, the highest productivity was 112.5 % and 128%, respectively, at concentrations of 11.2 mM (Fig 2 and Table S5). For the cell-free reactions based on

maltodextrin, those supplemented with trehalose and maltose (Fig 2F and 2H) showed a 112.5 % activity at 11.2 mM concentration, however it should be noted that the protein yields obtained using MDX in general represent about 50 % of those achieved with PEP (Fig 2K). Surprisingly, the addition of 13.7 mM lactose boosted protein production to 188 % (Fig 2I), allowing protein yields equal to those obtained with PEP (Fig 2K). A typical cell-free reaction uses simple substrate-level phosphorylation reactions to regenerate ATP using substrates with high-energy phosphate bonds such as PEP, acetate phosphate, glucose-6-phosphate (G6P), 3-phosphoglycerate, creatine phosphate (CP) or acetyl phosphate (AP)[9, 10]. However, the consumption of these compounds contributes to increased inorganic phosphate in the medium, which can eventually result in sequestration of free magnesium ions[43]. Protein synthesis can be inhibited due to the lack of these ions, which are needed for essential reactions such as nucleoside triphosphate synthesis and protein translation[44, 45]. An alternative, that avoids phosphate accumulation, is the use of MDX as a substrate for ATP regeneration. MDX is slowly metabolized in the cell-free mix and contributes to oxidative phosphorylation reactions, which recycle inorganic phosphate coming from other metabolic processes and from the specific phosphate donor (HMP) added in the reaction mixture. Consequently, phosphate accumulation is reduced, fluctuations in pH are lower and levels of ATP can be maintained for protein production[15, 16, 39]. This may help explain the observed beneficial impact of lactose on protein synthesis observed when MDX is used instead of PEP to energise reactions (Fig 2K). We speculate that lactose is consumed by β-galactosidase (induced by IPTG during cell-extract preparation) present in the cell-extracts[21], producing glucose as a secondary carbon source.

In order to further evaluate if lactose also acts as enhancer in dried samples containing MDX in their formulation, lyophilized and silica-dried reactions were made with 15 mM of sucrose (Table S3) or with a mixture of 15 mM sucrose and 15 mM lactose as protectants, and were rehydrated with 13.7 mM lactose or water respectively, after two weeks storage at room temperature. In contrast to earlier results in fresh samples supplemented with lactose (Fig 2I), we only observed a slight improvement in the cell-free reactions dried using the low-cost protocol (Fig S4). These results indicate that the enhancement due to lactose as an additive is only preserved in fresh cell-free reactions and not in the rehydrated samples, and effects on protein stability[46].

Linear DNAs can also act as templates for RNA and protein synthesis using cell-free technology. These DNA templates are a popular alternative to plasmids since their preparation is fast and convenient[47]. However, protein synthesis yields can be low due to endogenous exonuclease activity present in the cell-extracts, and degradation of DNA templates. To address this problem, strategies such as the use of Chi sequences[25], GamS [21, 47, 48]and PCR products with long flanks[3] have been used to stabilize linear DNAs. To determine whether the addition of lactose to either of our two fresh formulations (Tables S1-2) can improve the protein yields in cell-free reactions based on linear DNA templates, we tested the two best lactose concentrations from our previous experiments with fresh samples (Fig 2D, I and Fig S2I). In addition, linear DNA templates with T7 promoter and terminator were amplified with extended 100bp flanks to protect the template. We observed that extended flanking sequences stabilized linear templates in both cell-free formulations (Fig 3A and 3B), with the exception of those that were supplemented with Chi6 sequences or trehalose at higher concentration (11.2 mM) in the PEP formulation (Table S2). In the latter case, there was a slight improvement in the protein yields from 23 to 26 µM sfGFP when lactose was added, and the best performance (30 µM) was seen in samples treated with GamS. Surprisingly, the profile changed when maltodextrin was used in the reactions (Fig 3B). Addition of 13.7 mM of lactose showed the greatest yield improvement from 19 to 31 µM of sfGFP, even better that those obtained after supplementation with GamS (20 µM) or using plasmids as DNA templates in cell-free reactions based on this formulation (Table S1). Indeed, a similar boost in yields was seen when lactose was added to a commercial version of the cell-free extract (Linear DNA Expression Kit, MyTxTL 508024) (Fig S5), where maltodextrin is also used as an energy source in the commercial kit[19]. The addition of lactose provides a general boost for protein production

from linear DNAs protected with long flanks, achieving high protein titres (>30 µM sfGFP) in low-cost reaction mixtures (£0.044 per a 12 µL reaction) consuming maltodextrin as a cheap energy source.

Low-cost production of high value protein reagents

To test the utility of the expression systems described in this work, we expressed a modified version of *Bst* DNA polymerase[49] (Br512, Fig 4A) using the improved formulation with MDX (Table S1, cell-free formulation based on maltodextrin supplemented with 13.7 mM lactose). *Bst* is an isothermal polymerase commonly used in Loop-mediated isothermal amplification (LAMP) due to its high tolerance of clinical samples, and the enzyme is a useful component of rapid point-of-care diagnostic kits[50, 51]. The enzyme was produced by transcription-translation of a plasmid template in 20x 12µL reactions, followed by pooling of the samples and affinity-column purification of the protein product. This simple procedure yielded 60.9 ± 0.5 µg of Bst DNA polymerase and its activity was tested in a home-made colorimetric LAMP assay (Fig 4 B, top panel), displaying equal effectiveness to the equivalent commercial assay. The procedure allowed construction of a LAMP assay at a cost 20-fold cheaper than the commercial version. In addition, the approach reduced the need for specialised equipment, time and effort generally required to produce this polymerase[49, 52].

We had demonstrated that it was possible to produce sfGFP from linear PCR-amplified DNAs without the requirement for expensive reagents to protect the templates against exonucleases (Fig 3). We decided to use our lactose-containing extracts (Table S1, 13.7 mM lactose) to express BsaI, a type IIS restriction enzyme frequently used in Golden Gate cloning[53]. BsaI (EcoR31I) is an example of a toxic protein that can only be expressed in special *E. coli* strains that are protected by expression of the cognate methylase, or similar[54, 55]. For this reason, it is difficult to obtain plasmid DNA templates encoding the gene[56]. To side-step these problems, we used a chemically synthesised linear DNA template that was co-amplified with 4-oligonucleotides (Fig 4C; Table S11). The flanking oligonucleotides included a set of two adapters for the particular target sequence, and two longer sequences that could be reused (to avoid the costs of resynthesis). The final PCR product was 2043 bp size (Fig 4D), purified from 0.8 % (w/v) agarose gel and used directly as a template for protein production, as described for Bst DNA polymerase. To verify its activity, a restriction analysis was performed using the enzyme product (Fig 4E), confirming the feasibility of producing active reagents like Type IIS restriction enzymes through cell-free technology using synthetic dsDNA fragments that can be propagated by *in vitro* PCR.

To further challenge the system in the production of high value reagents, cell-free reactions were prepared through the low-cost drying method described above (TableS3-4) using PEP and MDX formulations and protected with 120 mM and 15 mM sucrose respectively. After two weeks of storage at room temperature, 20 x 12µL reactions were rehydrated with 5 nM of Br512 plasmid (for *Bst* expression) or 5 nM of linear DNA to produce BsaI. Lactose was not included in the reactions since the enhancer effect of lactose was only preserved in fresh reactions (Fig S4). After an overnight incubation at 29°C, this system yielded 37.0 ± 6.5 µg and 24.0 ± 0.7 µg of *Bst* DNA polymerase when PEP and MDX was used as energy source respectively. Using a home-made colorimetric LAMP assay (Fig 4B, bottom panel), the activity of Bst was tested showing a similar efficiency that when the polymerase was produced in fresh cell-free extracts. These results demonstrate the feasibility of producing protein reagents from dried cell-free reactions prepared by a low-cost drying system and using circular DNA as template. However, when linear DNA was used as template, the production of BsaI was not possible under the tested conditions even when a nuclease inhibitor (2 µM GamS) was added in the reaction, probably due to the instability of the linear DNA in the new reaction environment created after sample rehydration.

Deconstructing the cell-free formulation

To identify non-essential components in the cell-free mixture (Tables S7-8) and evaluate the enhancing effect of lactose in these reactions under fresh conditions, we successively removed each of the components present in the 25X Nucleotide mix (Table S7A) and 10X energy buffer (Table S8A), which are used to prepare cell-free mixtures based on PEP and MDX energy sources. We started with removal of the most expensive reagents, followed by elements thought to be essential[41]. Our analysis allowed us to identify three groups of reagents that we categorised as non-essential, beneficial, or essential for the cell-free reactions (Fig 5A). In the first group we observed a positive response when CoA, tRNA, NAD, Putrescine, cAMP (only included in the maltodextrin mix) were added in both formulations (Tables S7B-8B). Reactions supplemented with MDX showed a drop in the range of 10-17% in relative yields of sfGFP protein, which recovered when 13.7 mM lactose was added (Fig 5A). For the second group of reaction components, spermidine, CTP and GTP proved beneficial for both systems (Fig 5A). However, unlike the first group, yields did not recover on addition of lactose. The removal of UTP or folinic acid from cell-free reactions based on maltodextrin resulted in the plunge of protein synthesis yields to 4 %, and total loss in the absence of ATP. Surprisingly, sfGFP protein yields remained at 50 % when lactose (11.2 mM) was added to the PEP-based reactions (Fig 5A and 5B). Further, we did not observe a full loss of protein synthesis after completely removing all these components, and the addition of lactose improved the yield from 29 to 43 % (Fig 5A and 5C). A possible explanation is that the conversion from PEP to pyruvate is coupled to nucleotide regeneration and mRNA translation [43, 57]. To investigate this, the concentrations of NTPs, NMPs and pyruvate were measured by LC-MS (Table S12, Fig S6) in those samples devoid of external sources of nucleotides but fed either with PEP or MDX. Our results demonstrated that the ability to regenerate nucleotides (NMPs, NTPs) and pyruvate in cell-free reactions is related with the addition of an energy source since when PEP is removed from the mixture (Fig S7A), the concentration of these metabolites was depleted. In contrast, when PEP was added into the mixture, our results indicated endogenous biosynthesis of NTPs (Fig 5D, upper panel) during the CF reaction due to pyruvate formation (Fig 5D, lower panel), while high levels of GTP (18.69 µM) and ATP (12.43 µM) were seen. This is consistent with the requirement for these nucleotides in mRNA translation, where two GTPs are required for each cycle of aminoacyl-tRNA delivery and ribosome translocation, and one ATP is required for peptide bond formation[13, 57]. Interestingly, the addition of MDX (supplemented or not with lactose) in CF reactions also sustains NTP and pyruvate biosynthesis (Fig 7SB), recording higher levels of GTP (30.6 µM) and ATP (44.9 µM) than in the PEP formulation when lactose and MDX are included in the reaction. This is consistent with our previous results (Fig 3B), where protein production is enhanced in cell-free reactions based on MDX and supplemented with lactose as additive. However, it does not explain the observed inability of maltodextrin to sustain protein production when exogenous nucleotides are omitted from the reactions (Fig 5A). We speculate the glycolytic pathway and nucleotide pool were affected by lack of crowding agents and other additives missing from the maltodextrin system (Table S8), which may delay the synthesis of NTPs, since in the PEP system the maximum GTP concentration was observed at after 5 h of incubation, while in MDX formulation the maximum was registered after 15 h (Fig 5D, S7B). In addition, the fast consumption of pyruvate and the absence of some NMPs such as GMP in the CF reactions supplemented with MDX, seems to compromise the activity of PANOx system which is coupled to maltodextrin system [15, 58]. In fact, an increment in the AMP concentration and a reduction in the ATP levels were measured, suggesting a reconversion from the triphosphate to monophosphate form (Fig S7B), which agrees with previous studies where cell-free systems based on glucose as energy source and fed with NMPs did not result in protein synthesis, but showed conversion of ATP to AMP [59]. Accordingly, protein synthesis was only possible in the PEP system, where, with the enhancing effect of lactose, production of 16.92 µM sfGFP was observed.

Overall, our combined findings suggest that it is possible to reduce the cost of cell-free formulations (Table S14), especially for use in an educational context or other low-resource settings, since the

activities of these simpler extracts are high enough for detection in the classroom (Fig 5B). So far, we have achieved an over 400-fold reduction in reaction costs compared to commercial versions and almost 3-fold reduction compared to the cost of DIY reactions (Fig S8). This is due to the use of cheaper sources of PEP (22.2 times less expensive than other suppliers (Fig S8A-B, D-E), and savings due to the removal of the nucleotide mix, which represents 45.84 % (Fig S8A and Fig S8F) in the total cost of a reaction. Our study demonstrated a cell-free formulation completely free of external NTPs, which is even cheaper than maltodextrin system using PEP from a different supplier (Fig S8D-E). This extends previous attempts to build economical and open methods for programmable biosynthesis[11, 18, 60-63].

CONCLUSIONS

Cell-free technologies offer many potential uses for education, research and point-of-care and field applications in LMICs, and numerous efforts have been made to improve access to these tools by removing cost barriers in manufacturing[2, 11, 16, 18, 64]. Despite much progress, improvements are still required. Here we describe several advances. First, we developed a cost-effective platform (USD $177) based on silica beads and a conventional low-vacuum source to dry cell-free reactions without use of expensive equipment (>USD $10 000). Using this platform, we achieved up to 19 µM sfGFP protein production after two weeks of storage at room temperature. While the use of silica for drying cells has been previously reported[65], here we report the first use for preserving fully assembled cell-free reactions ready for cold chain-free distribution and use. Second, we describe the enhancing effect of lactose in cell-free formulations, obtaining a substantial improvement in reactions that use maltodextrin as an energy source. This improvement allowed us to use lactose as an additive for expressing proteins using linear DNA templates without the addition of costly stabilisers such as GamS. Finally, we demonstrated that protein synthesis is sustainable in cell extracts without adding an external source of NTPs. We believe that this is the forerunner of future work to more deliberately exploit regeneration systems in cell-free reactions, to further lower costs and pave the way for wider use of these systems in low-resource contexts.

METHODS

Molecular biology

Unless otherwise stated, all PCR reactions were performed using Q5 High-Fidelity 2X Master Mix (New England Biolabs, M0492S) according to the manufacturer's instructions. For a single PCR reaction using 4 primers simultaneously, the reaction mix was composed of 0.5 µM of each adapter primer, 0.025 µM each core primer (20X less concentrated than adapter primers), 40-60 ng of DNA template and 1X Q5 High-Fidelity Master Mix. PCR conditions are described in Table S10. Cell-free backbone plasmid was synthesized by IDT (Integrated DNA Technologies, USA) using kanamycin as resistance marker. New plasmids were constructed using conventional PCR products or double-stranded DNA fragments (Genewiz, UK) along with the destination pFGC-T7-RJBB plasmid (vector backbone with T7 promoter-RiboJ- BsaI-LacZα-BsaI-T7 terminator configuration) in a single Golden Gate cloning reaction. All the molecular cloning steps and plasmid propagation were performed in *E. coli* Top10 (Invitrogen, C404010). DNA plasmid for cell-free reactions was obtained by midi-prepping (Sigma, NA0200-1KT) an overnight culture of 50 mL LB with the appropriate strain and antibiotic according to the fabricant's instructions. Plasmids and primers are listed in Table S9 and S11 respectively. Coding sequences cloned into pFGC-T7-RJBB plasmid are described in Table S12. Plasmids are available at Addgene.

Extract preparation

To prepare crude cell-extracts, 5 µL of BL21 Star glycerol stock (Invitrogen, C601003) was inoculated into 5 mL of 2xYT medium. The preculture was grown for 8h at 37°C with 200 rpm shaking. Afterwards, 50 mL of 2xYT medium was inoculated with 30 µL preculture in a 250 mL flask and grown at 37°C with vigorous agitation (200 rpm). The next day, the stationary phase preculture was used to inoculate 400 mL 2xYT media supplemented with 18 g/L D-g glucose in a 2.5 L baffled Tunair flask (Sigma Aldrich, Z710822), giving an initial optical density (OD600) of 0.05. Cultures were grown at 37°C with shaking (200 rpm) until OD600 reached 0.5 (approximately 2.5 h), then the cells were induced with 400 µL of 1 M IPTG. Cells were harvested in the exponential phase at an optical density (OD600) of 2.0 by centrifugation at 5000 g and 4 °C for 12 min. Pellets were washed three times with S30A buffer (50 mM Tris base, 14 mM magnesium glutamate, 60 mM potassium glutamate, 2 mM dithiothreitol (DTT), pH 7.7 adjusted with 1:1 acetic acid). After, the pellet was weighed and resuspended in 0.9 mL of S30B buffer (5 mM Tris base, 14 mM magnesium glutamate, 60 mM potassium glutamate, 1 mM dithiothreitol, pH 8.2 adjusted with 1:1 acetic acid) per gram of pellet. Cell suspension was distributed in 1 mL aliquots in 1.5 microcentrifuge tubes and then lysed by sonication on a QSonica Q125 sonicator with a 3.175 mm diameter probe as previously described by Silverman et al.[66]at a frequency of 20 kHz and 50% amplitude by 10 s ON/OFF pulses for a total of 60 s (delivering ∼350 J). The lysate was centrifuged for 10 min at 4°C and 10 000 g. To clarify the cell-extracts, the supernatants were centrifuged a second time for 15 min at 4°C and 12 000 g. Finally, the crude extracts were pooled, supplemented with 1 mM DTT, aliquoted and snap-frozen in liquid nitrogen.

Cell-free reactions

A typical cell-free reaction based on PEP as energy source was composed of 175 mM potassium glutamate, 10 mM ammonium glutamate, 2.7 mM potassium oxalate, 1 mM putrescine, 1.5 mM spermidine, 0.33 mM NAD, 1.2 mM ATP, 0.86 mM CTP, GTP and UTP, 0.27 mM CoA, 0.172 mg/mL of MRE600 *E. coli* tRNA, 0.07 mM folinic acid, 33 mM PEP, 2 mM of each of the 19 amino acids (glutamate was omitted since it is already present in the reaction mixture as potassium glutamate), 10 mM magnesium glutamate, 2%(w/v) PEG-8000, 5nM DNA (plasmid or linear) and 33.33% (v/v) of crude extract by volume. For reactions using maltodextrin (MDX) as energy source, the cell-free mixture is composed of 50 mM HEPES pH 8, 1.5 mM ATP and GTP, 1.4 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM CoA, 0.33 mM NAD, 0.76 mM cAMP, 0.01 mM folinic acid, 0.11 mg/mL spermidine, 2% (w/v) PEG-8000, 3.4 mM of each of the 19 amino acids, 12 mg/mL maltodextrin, 0.60 mg/mL sodium hexametaphosphate, 2.6 mM magnesium glutamate, 56 mM potassium glutamate, 5 nM DNA (plasmid or linear DNA) and 33.33 % (v/v) of crude extract by volume. An Ultra low-cost cell-free reaction contains 175 mM potassium glutamate, 10 mM ammonium glutamate, 2.7 mM potassium oxalate, 33 mM PEP, 2 mM of each of the 19 amino acids, 10 mM magnesium glutamate, 2% (w/v) PEG-8000, 5 nM DNA (plasmid or linear), 11.25 mM lactose as enhancer and 33.33% (v/v) of crude extract by volume. Detailed protocols for preparing all cell-free stock solutions used in this study are available at protocols.io/researchers/fernando-guzman-chavez.

Lyophilization and silica-drying of cell-free reactions

Unless otherwise specified, all the lyophilization mixes contain the composition described above for cell-free reactions using either PEP or MDX as energy source, excluding the DNA and adding the different cryoprotectants at the tested concentrations (0, 5, 15, 30, 60 and 120 mM) in the lyophilization mix (refer to Tables S1-6 for more details in the cell-free reactions composition). This mix was distributed in 96 well PCR plates (4titude, 4ti-1000/R) in 9 µL aliquots for mixes containing PEP and 11 µL aliquots for those with MDX. Using different wells in the same plate, 20 nM psfGFP plasmid[17] was distributed in 9 µL volumes along the plate. Afterwards, the 96 well plate was sealed with adhesive aluminium foil seals (4titude, 4ti-0550) and punctured with a 16G needle to create one

hole. For lyophilisation, the samples were frozen at -80°C for 30 min and then placed to a FreeZone Triad Benchtop Freeze Dry System (Labconco), previously cooled reaching a condenser temperature of −80 °C. Then, the samples were freeze-dried following a three-step program: 12 h at -45°C, 10 h at -5°C and 4 h at 20°C with a constant pressure of 0.04 mbar throughout the process. The temperatures indicated in the three-step program correspond to shelf temperatures inside of the chamber.

For low-cost drying, the samples were transferred to low-cost drying device (Fig 1A), which consisted of a Nalgene Desiccator (ThermoFisher, 5311-0250PK) with 500 g of silica gel (Fisher Scientific, S/0761/53) connected to the laboratory vacuum system. The samples were left to dry overnight at room temperature under vacuum. Next day, the plate was sealed with adhesive aluminium foil seals (4titude, 4ti-0550) and punctured with a 16G needle to create one hole.

In both cases and unless rehydrated immediately, freeze-dried reactions were packaged as previously described by Jung, J.K et al 2020 [23] with the following modifications: the dry samples were placed into a vacuum sealer bag (12 cm x 16 cm Vacuum Food Sealer Embossed Bags, Amazon, Amazon Standard Identification Number (ASIN) B015A7LH9A) with two desiccant packs (2 g Small Silica Gel Sachets, Amazon, ASIN: B07PRGC434), two oxygen absorbers (Fresherpack 20cc Oxygen Absorbers, Amazon, ASIN: B00U2O3VAK), purged with argon using an argon canister (Preservintage Wine Preserver, Amazon, ASIN: B07MQFTKPN) and impulse heat sealed (Audew Food Vacuum Sealer, Amazon, ASIN: B07QC2BTJ9). Afterwards, the samples were placed in a second a light-protective bag (Open Top Mylar Foil Aluminium Bag, Amazon, ASIN: B01MY95ICS) and impulse heat sealed.

Fluorescence quantification

Fresh cell-free reactions were prepared as described in Tables S1-S2. Dried samples of DNA plasmid were rehydrated with 36 µL of PCR grade sterile water (MQ) in order to produce a concentration of 5 nM. Cell-free pellets were reconstituted with 12 µL of the plasmid solution and incubated at room temperature for 1 min. According to the experiment, ten microliters of either fresh or rehydrated samples were loaded into V-bottom 96 well plates (Corning, CLS3957). Reactions were incubated in a CLARIOStar plate reader (BMG Labtech, Germany) at 29°C and fluorescence measurements (emission/excitation: 470/515 nm; gain = 500) were recorded every 6 min for 18 h. To quantify fluorescent protein concentrations, recombinant eGFP standard (Cell Biolabs, STA-201) was used to create a calibration curve.

Cell-free reactions using linear DNA

Cell-free reactions were set up as described above. When required, GamS and Chi6 (exonuclease inhibitors) were added to a final concentration of 2 µM [48]while the corresponding volume of water in the cell-free reaction mix was adjusted (Table S3-4). Linear templates were prepared by PCR amplification and purified from 0.8 % (w/v) agarose gel using Monarch DNA Gel Extraction Kit (New England Biolabs, T1020S), according to manufacturer's protocol. When psfGFP plasmid was used as template, purified PCR products were treated with FastDigest DpnI restriction enzyme (Thermo Scientific, FD1704) for 30 min at 37°C to cut methylated DNA in order to eliminate the DNA template. After, the samples were purified using Monarch PCR & DNA Gel Cleanup Kit (New England Biolabs, T1030S) and quantified using a nanodrop spectrophotometer (Thermo Scientific, NanoDrop One).

Removing reagents in cell-free reactions

To study the effect of different constituents in the cell-free composition, 25X Nucleotide Mix and 10X Energy were modified according to Table S7-S8. In all cases, omitted reagents were replaced with the equivalent volume of MQ water and adjusted to pH 7.5. To perform either a cell-free reaction based

on PEP or MDX, the modified versions were used to prepare a 4X wizard mix or 2.5X reaction buffer instead of the complete version. Reactions were assembled according to Table S1-2. All the reactions were incubated at 29°C and measurements were recorded every 6 min for 18 h.

Cell-free production of BsaI enzyme

To produce BsaI enzyme, 20 cell-free reactions (12 µL per reaction) based on maltodextrin and supplemented with lactose (13.7 mM, Table S1 and S5) were performed using as DNA template the PCR product amplified from a double-stranded DNA fragment (gBlock, Table S12), purified from 0.8 % (w/v) agarose gel and incubated overnight at 29°C. To generate the PCR fragment, a 4 oligos PCR approach was performed as above described. Next day the samples were pooled, diluted 1:1 with 100 % glycerol and stored at -20°C. To test the activity, 400 ng pGEX-ilux plasmid was digested in home-made CutSmart® Buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium acetate, 100 µg/ml BSA, pH 8) at 37°C for 1 h. Digested plasmid was cleaned using a Monarch PCR & DNA Cleanup kit (NEB, T1030S) following the manufacturer's instructions and visualized in a 0.8% (w/v) agarose gel. Undigested plasmid was used as control.

LAMP colorimetric assay

To perform LAMP assays, Br512 (a modified version of B*st* DNA polymerase) was produced using cell-free technology. In short, 20 reactions (12 µL per reaction) were performed using the plasmid pKAR2-Br512[49](Addgene: 161875) as DNA template either in a fresh cell-free mixture based on maltodextrin and supplemented with lactose (13.7 mM, Table S1 and S5) or in rehydrated samples after two weeks of storage at room temperature. Dry samples were prepared through the low-cost drying system as described above, using PEP and MDX as energy source and protected with sucrose (120 mM and 15 mM respectively). Reactions were incubated overnight at 29°C. Next day, the 20 reactions were pooled and purified using a Ni-NTA Spin Column (Qiagen, 31314) according to the manufacturer's instructions. The sample was eluted in 300 µL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8) and diluted by adding 200 µL of buffer A (50 mM NaH_2PO_4, 50 mM NaCl). Afterwards, the sample was concentrated, and buffer exchanged using a 3K Amicon filter (Merck, UFC500324) with storage buffer (50 mM NaH_2PO_4, 50 mM NaCl, 2 mM DTT, 0.2 mM EDTA, 0.2 % Triton X-100, pH 8). Purified protein from 20 cell-free reactions (12µL per reaction) was quantified using Pierce 660 nm Protein assay (Thermo Scientific, 22660), visualized in a coomassie blue polyacrylamide gel and then diluted 1:1 with 100% glycerol and stored at -20°C. LAMP colorimetric reactions were prepared in 25 µL volume containing 1X colorimetric buffer[67] (10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween 20, 0.1 mM Cresol Red, pH 8.8), DNA template (Actin B, Table S12), 6 mM $MgSO_4$, 1.4 mM dNTPs, Br512 purified protein (10 pmoles), 1.6 µM each FIP and BIP primers, 0.2 µM each F3 and B3 primers, 0.4 µM each loop primers (Table S11). Reactions were incubated at 65°C for 30 min in a thermocycler (Applied Biosystems, ProFlex PCR system). WarmStart® Colorimetric LAMP 2X Master Mix (New England Biolabs, M1800S) was used as control to evaluate the efficiency in the colorimetric LAMP assay.

Quantification of nucleotides and pyruvate by LC-MS

To quantify nucleotides and pyruvate, the equivalent volume of 20 cell-free reactions (12µL per reaction) were prepared as described in Table S13, replacing the indicated volume of DNA with MQ water. CF reactions were supplemented with 11.2 mM lactose as indicated. Samples were incubated for 0, 0.5, 5 and 15 h at 29°C, then were analysed by LC-MS using the protocol described in Vilkhovoy et al. [68] Briefly, the samples were first deproteinized by adding an equal volume of ice-cold 100%

ethanol. This mixture was centrifuged at 12,000 x g for 15 min at 4°C, and the supernatant fraction, which contained the metabolites, was collected and diluted five-fold in ultrapure water to a volume of 50 µL. To tag the samples with aniline, 5 µL each of EDC (200 mg/mL) and 12C aniline were added to the mixture (13C in the case of internal standards), and the reaction was mixed at room temperature for 2 hours by gentle shaking. The tagging reaction was quenched by adding 1.5 µL of triethylamine and centrifuging the mixture at 13,500 x g for 3 min. 25 µL each of the tagged internal standard and tagged samples were mixed, and then analysed by the LCMS system. LC separation was performed on an Acquity BEH C18 Column (1.7 µm, 2.1 mm × 150 mm) at a flow rate of 0.3 mL/min and an injection volume of 5 µL. The elution started from 95% mobile phase A (5 mM tributylamine (TBA) aqueous solution, adjusted to pH 4.75 with acetic acid) and 5 % mobile phase B (5 mM TBA in acetonitrile), raised to 70 % B in 10 min, further raised to 100 % B in 2 min, held at 100 % B for 2 minutes, returned to initial conditions over 0.1 min, and held for 4 min to re-equilibrate the column. The mass spectrometer was set to negative ion mode with a probe temperature of 520 °C, negative capillary voltage of -0.8 kV, positive capillary voltage of 0.8 kV, and an acquisition range of m/z 130 - 900.

ASSOCIATED CONTENT

Supporting Information

Figure S1: Lyoprotectant effects of five sugars individually added to the two cell-free formulations. Figure S2: Lyoprotectant effects of five sugars individually added in higher concentrations. Figure S3: Sharing lyophilised and dried cell-free reactions around the globe. Figure S4: Effect of lactose in cell-free reactions before and after being added during the lyophilisation/drying process. Figure S5: Enhancing effect of lactose over sfGFP production in fresh cell-free reactions in three different formulations (PEP, MDX or commercial version). Figure S6: Chromatograms of NTPs and pyruvate detection by LC-MS at four time points. Figure S7: Regeneration of NMPs, NTPs and pyruvate during the cell-free reactions. Figure S8: Cost comparison between five different cell-free formulations.
Table S1: Composition of fresh MDX-based energy mix. Table S2: Composition of fresh PEP-based energy mix. Table S3: Composition of MDX-based energy mix for lyophilisation. Table S4: Composition of PEP-based energy mix for lyophilisation. Table S5: Sugar concentrations used in fresh and lyophilised extracts. Table S6: Source of sugars used in this study. Table S7: Composition of 25x nucleotide mix variants. Table S8: Composition of 10x nucleotide mix variants. Table S9: Plasmids used in this study. Table S10: PCR conditions. Table S11: Primers used in this study. Table S12: Sequences used in this study. Table S13: Ultra-low-cost (ULC) formulation for fresh PEP-based cell-free reactions.

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Author Contributions

FGC designed the study, performed the experiments, wrote the manuscript and carried out the data analysis. JH conceived the study, supervised and coordinated the design, interpreted the data and corrected the manuscript. AA and FF performed the experiments in Chile. AAdhikari,SV and JDV supported mass spectrometry. JAPG helped to draft the manuscript. JAPG and SSN performed the experiments in Mexico. JM and CG helped in the experimental design. JWA contributed to the coordination of the project.

ACKNOWLEDGMENTS

The authors acknowledge the funding support from EPRSC (EP/R014000/1, LCVD: Low-cost Cell-extract Viral Diagnostics, FGC was supported by LCVD project; BBSRC/EPSRC OpenPlant Synthetic Biology Research Centre Grant BB/ L014130/1 to JH. The authors acknowledge Dr. Khalid K. Alam, former postdoc at Northwestern University who shared tips and protocols on preservation strategies after lyophilization. FF was funded by Fondo de Desarrollo de Áreas Prioritarias - Center for Genome

Regulation (ANID/FONDAP/ 15090007). FF and AA were founded by Proyecto Investigación Interdisciplinaria 2018 VRI. AA was funded by ANID National Doctoral Scholarship ID:21140714 , and ANID - Millennium Science Initiative Program - ICN17_022, ICGEB CRP/CHL19-01. AAdhikari and SV were supported by the Center on the Physics of Cancer Metabolism at Cornell University through Award Number 1U54CA210184-01 from the National Cancer Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. Funding from CONACyT A1-S-17269, DGAPA-PAPIIT IN225220 to SSN.

FIGURE LEGENDS

Figure 1. Drying systems to preserve active cell-free extracts. A) Low-cost drying: Low-vacuum, room temperature drying apparatus using silica beads. B) High-cost lyophilization: Commercial, computer-controlled freeze dryer for lyophilization (FreeZone Triad Benchtop Freeze Dry System (Labconco). C) Percentage of recovery after 1 day and 2 weeks of storage at room temperature in sample. Cell-free reactions based on PEP (top panel) or MDX (bottom panel) as energy sources. Plasmid psfGFP was used as DNA template. Percentage of recovery was calculated relative to the RFU value obtained from fresh extracts with the respective energy sources (PEP or MX). Cell-free reactions were incubated at 29°C for 15h. Error bars represent standard error over twelve technical measurements.

Figure 2. Lyoprotectant effects of five sugars individually added to the two cell-free formulations. CFPS based on PEP (A-E) and MDX (F-J) and dehydrated either by high-cost lyophilization or the low-cost drying method. Samples were dried and stored at room temperature for 1 day and 2 weeks. Cell-free reactions were rehydrated and incubated at 29°C for 15h. The final concentrations of additives in the reactions are indicated on the horizontal axes. The percentage of recovered protein production was calculated relative to that seen in fresh, additive-free reactions with the energy sources PEP or MDX. Error bars represent standard error over three technical measurements. K) Effects of lactose on protein yields in fresh cell-free reactions based on PEP or MDX as energy source. Samples were supplemented with lactose, 11.2 mM (PEP mixture, Table S2, Table S5) and 13.7 mM (MDX mixture, Table S1, Table S5) as indicated. Cell-free reactions for production of green fluorescent protein were incubated at 29°C for 15h using psfGFP as DNA template. Yields were calculated relative to fluorescence values seen in PEP formulated cell-free reactions in the absence of lactose. Error bars represent standard error over three technical measurements.

Figure 3. Enhancer effect of lactose on gene expression using linear DNA templates in cell-free reactions. CFPS based on A) PEP or B) MDX. Details of additives in each cell-free mixture are shown in Table S5. GamS and Chi6 were added at a final concentration of 2 µM. Except for NTC (no template control), all reactions contained 5 nM DNA (plasmid or linear). Linear DNA templates were amplified with extended 100bp flanks to protect the template (highlighted with a grey dashed box). Unprotected linear DNA templated was amplified with extended 3 bp flanks (denoted as "short flanks"). Black strips are representative images of the fluorescence signal on the plate captured using an imaging system (BioRad GelDoc-Go). Cell-free reactions were incubated at 29°C for 10h. All error bars represent standard error over three biological replicates based on three technical measurements.

Figure 4. Production of molecular biology reagents. A) Purified Br512 Bst DNA polymerase) visualized in a polyacrylamide gel stained with coomassie blue. B) Colorimetric LAMP assay using the Br512 Bst DNA polymerase produced *in vitro* in both fresh conditions (top panel) and using rehydrated samples (bottom panel) after two-week storage at room temperature. Cell-free reactions based on PEP and MDX were prepared using the low-cost drying system and protected with sucrose (120 mM and 15 mM respectively). A synthetic dsDNA fragment from actin B gene (*Homo sapiens*) was used as target at the following amounts: 0, 0.025,0.25,2.5,250 and 2500 pmoles. Primers used in this assay are

described in Table S11. Negative reactions were pink-coloured and positive reactions changed to yellow. C-D) A PCR product encoding the BsaI restriction endonuclease (2043 bp) was amplified using a single PCR with 4 oligonucleotides. An inner set of core primers provided a template for secondary amplification by longer oligonucleotides. The resulting product had extended terminal sequences that helped protect the coding region from exonuclease degradation. E) Testing of BsaI by restriction endonuclease digestion of luxpGEX plasmid. Digestion was performed using BsaI produced by cell-free technology. Plasmid DNA samples were treated with 1) FastDigest Eco31I (Thermo Scientific, FD0293) (Isoschizomer: BsaI), 2) BsaI in Cell-extract, 3) BsaI in Cell-extract: 100% glycerol (1:1). Expected size of bands after digestion: 6440 and 4433 bp.

Figure 5. Ultra-low-cost (ULC) cell-free formulation based on PEP. A) Relative levels of cell-free protein synthesis after successive removal of reaction components according to tables S7B and S8B. Every sequential row removes one more reagent in addition to the ones above it. Cell-free reactions were prepared with PEP or MDX as an energy source. The importance of additional components in the reaction buffers was tested by omission, starting with the most costly and less essential. The activities of the cell free extracts were measured by sfGFP production, and normalised relative to the respective full reaction (PEP/PEP complete reaction or MDX/MDX complete reaction). All measurements were based on three biological and three technical replicates. Relative level of protein synthesis for the ultra-low-cost (ULC) cell-free formulation is highlighted with a black square. B) Synthesis of fluorescent proteins using the ULC cell-free formulation supplemented with 11.25 mM lactose (Table S13). Reporters: 1) psfGFP, 2) pJL1-eforRed, 3) pJL1-dTomato, 4) pFGC-T7-RibJ-mScarlet, 5) pFGC-T7-RibJ-RRvT, 6) pFGC-T7-RibJ-mTFP1. C) Quantification of sfGFP production in ULC-PEP formulation supplemented with 11.25 mM lactose. D) Regeneration of NTPs, NMPs and pyruvate during the cell-free reactions based on ULC-PEP formulation, measured by LC-MS at four time points. Samples were prepared as described in Table S13, replacing the indicated DNA with MQ water. Cell-free extracts were supplemented with 11.25 mM lactose where is indicated. Concentrations of nucleotides and pyruvate were measured by LC-MS.

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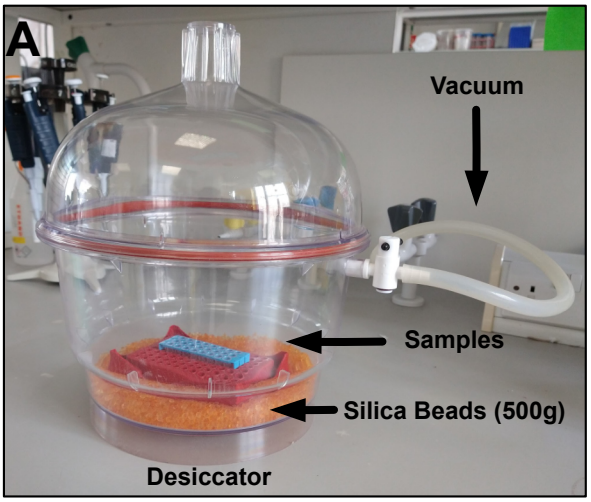
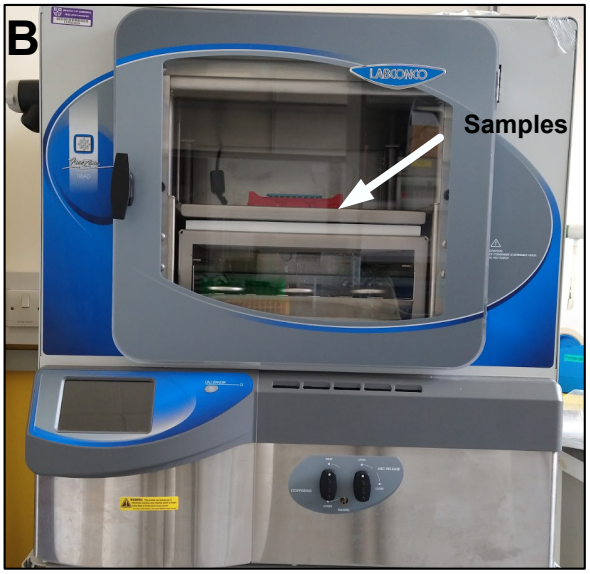
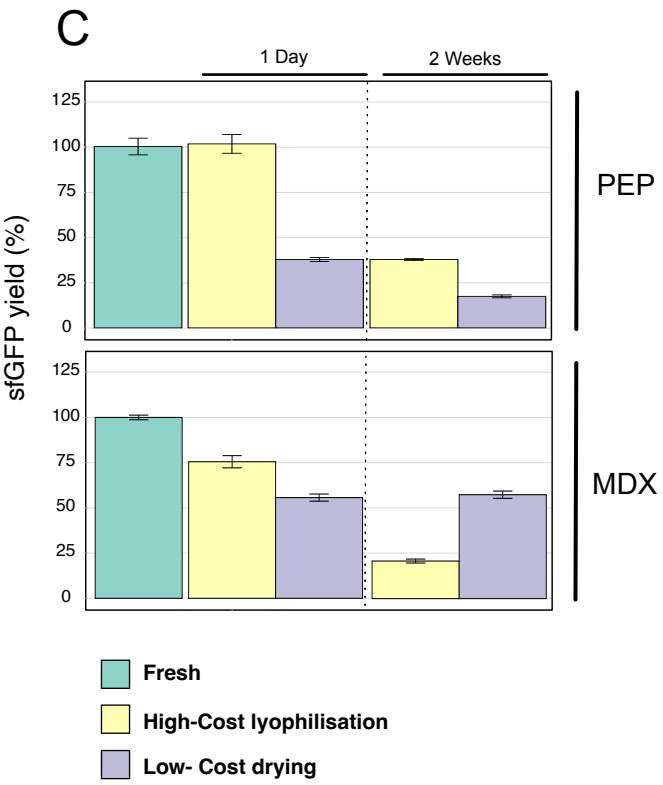
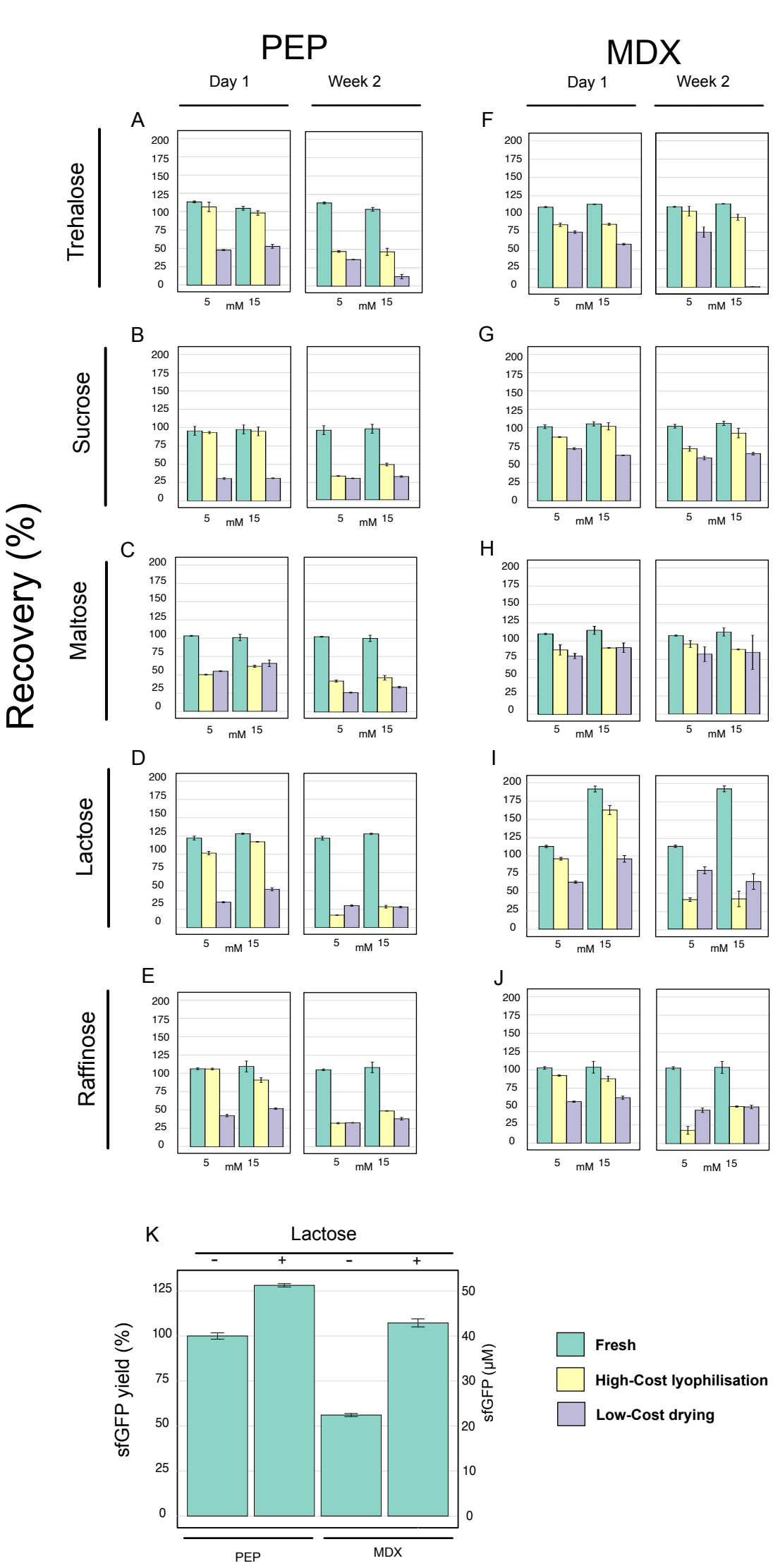
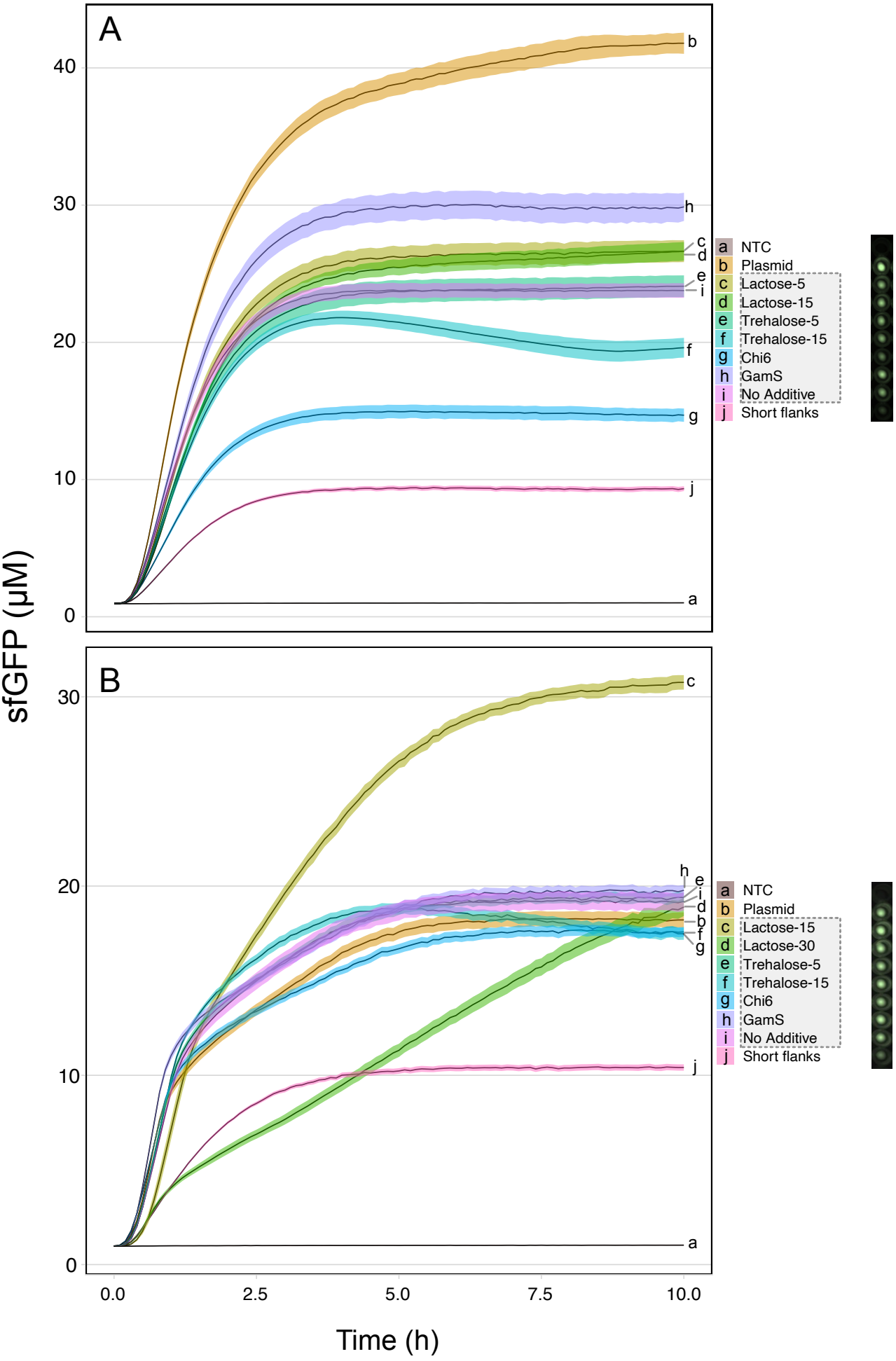
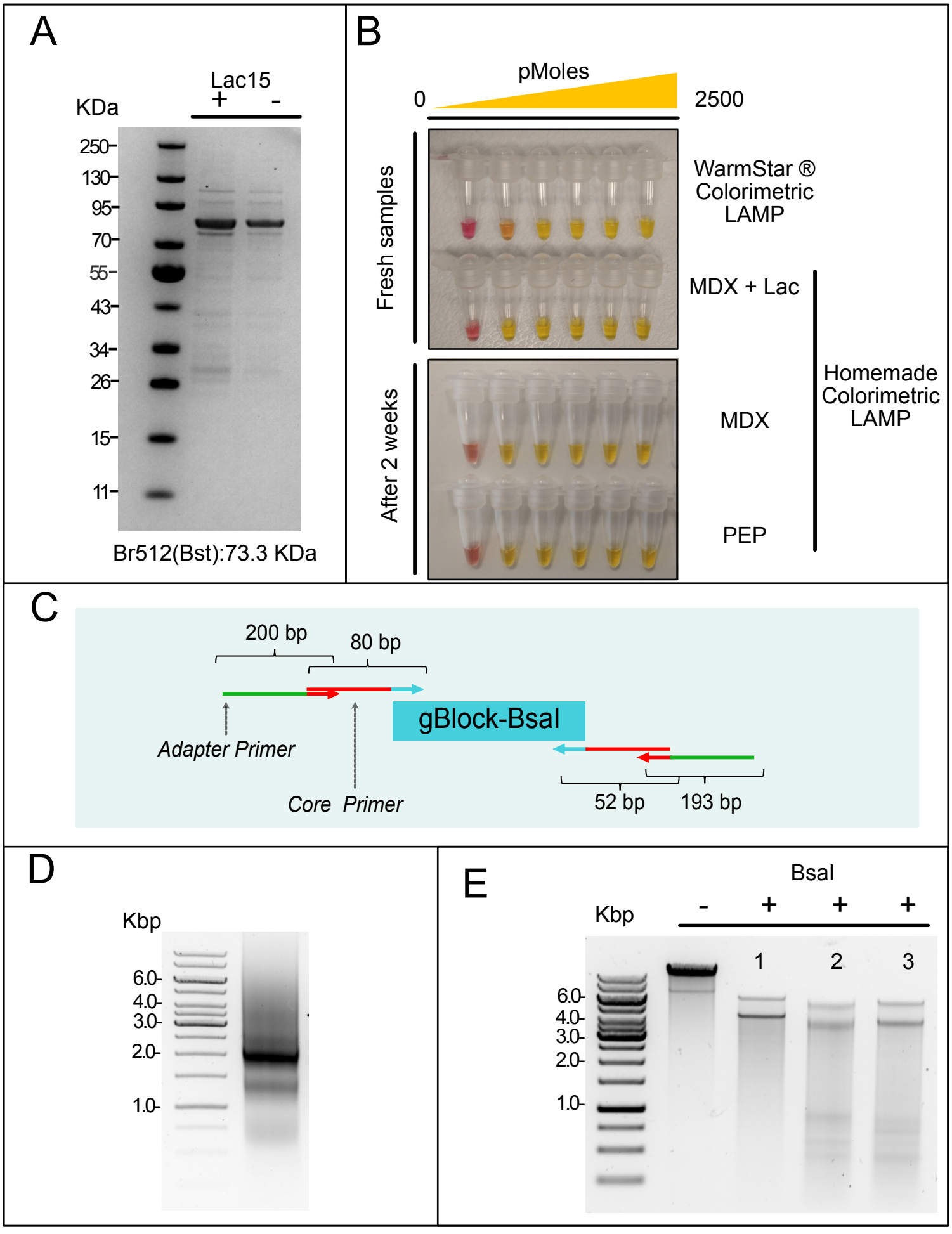

A
Vacuum
Samples
Silica Beads (500g)
Desiccator

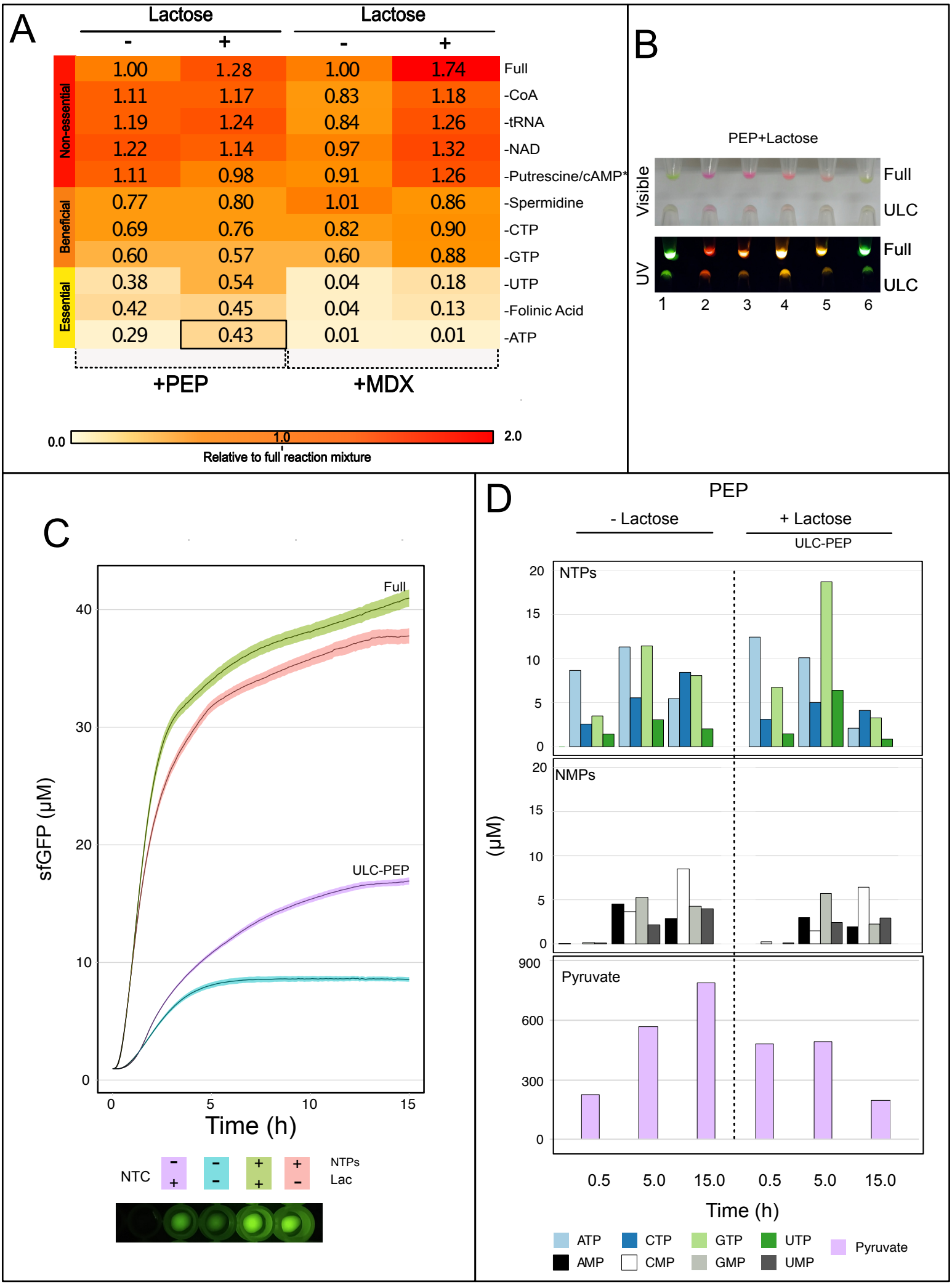

B
LABCONCO
Samples


C
1 Day
2 Weeks
sfGFP yield (%)
125
100
75
50
25
0
PEP
MDX
Fresh
High-Cost lyophilisation
Low- Cost drying


PEP
MDX
Day 1
Week 2
Day 1
Week 2
Recovery (%)
Trehalose
Sucrose
Maltose
Lactose
Raffinose
A
B
C
D
E
F
G
H
I
J
200
175
150
125
100
75
50
25
0
5
mM
15
K
Lactose
-
+
-
+
sfGFP yield (%)
sfGFP (µM)
125
100
75
50
25
0
50
40
30
20
10
0
PEP
MDX
Fresh
High-Cost lyophilisation
Low-Cost drying


A
B
sfGFP (μM)
Time (h)
0
10
20
30
40
0.0
2.5
5.0
7.5
10.0
a NTC
b Plasmid
c Lactose-5
d Lactose-15
e Trehalose-5
f Trehalose-15
g Chi6
h GamS
i No Additive
j Short flanks
a NTC
b Plasmid
c Lactose-15
d Lactose-30
e Trehalose-5
f Trehalose-15
g Chi6
h GamS
i No Additive
j Short flanks


A
Lac15
+ -
KDa
250
130
95
70
55
43
34
26
15
11
Br512(Bst):73.3 KDa
B
pMoles
0
2500
Fresh samples
After 2 weeks
WarmStar ®
Colorimetric
LAMP
MDX + Lac
MDX
PEP
Homemade
Colorimetric
LAMP
C
200 bp
80 bp
gBlock-BsaI
Adapter Primer
Core Primer
52 bp
193 bp
D
Kbp
6.0
4.0
3.0
2.0
1.0
E
BsaI
Kbp
- + + +
1
2
3
6.0
4.0
3.0
2.0
1.0


A
Lactose
-
+
Lactose
-
+
Non-essential
Beneficial
Essential
1.00 1.28 1.00 1.74 Full
1.11 1.17 0.83 1.18 -CoA
1.19 1.24 0.84 1.26 -tRNA
1.22 1.14 0.97 1.32 -NAD
1.11 0.98 0.91 1.26 -Putrescine/cAMP*
0.77 0.80 1.01 0.86 -Spermidine
0.69 0.76 0.82 0.90 -CTP
0.60 0.57 0.60 0.88 -GTP
0.38 0.54 0.04 0.18 -UTP
0.42 0.45 0.04 0.13 -Folinic Acid
0.29 0.43 0.01 0.01 -ATP
+PEP
+MDX
0.0
1.0
2.0
Relative to full reaction mixture
B
PEP+Lactose
Visible
UV
Full
ULC
Full
ULC
1 2 3 4 5 6
C
Full
ULC-PEP
sfGFP (μM)
Time (h)
0 10 20 30 40
0 5 10 15
NTC
NTPs
Lac
D
PEP
- Lactose
+ Lactose
ULC-PEP
NTPs
NMPs
Pyruvate
(μM)
0 5 10 15 20
0 300 600 900
0.5 5.0 15.0 0.5 5.0 15.0
Time (h)
ATP CTP GTP UTP
AMP CMP GMP UMP
Pyruvate