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ARTICLE

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# Unexpected instabilities explain batch-to-batch variability in cell-free protein expression systems

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## Abstract

Cell-free methods of protein synthesis offer rapid access to expressed proteins. Though the amounts produced are generally only at a small scale, these are sufficient to perform protein-protein interaction assays and tests of enzymatic activity. As such they are valuable tools for the biochemistry and bioengineering community. However the most complex, eukaryotic cell-free systems are difficult to manufacture in house and can be prohibitively expensive to obtain from commercial sources. The *Leishmania tarentolae* system offers a relatively cheap alternative which is capable of producing difficult to express proteins, but which is simpler to produce in large scale. However, this system suffers from batch-to-batch variability, which has been accepted as a consequence of the complexity of the extracts. Here we show an unexpected origin for the variability observed and demonstrate that small variations in a single parameter can dramatically affect expression, such that minor pipetting errors can have major effects on yields. *L. tarentolae* cell-free lysate activity is shown to be more stable to changes in $Mg^{2+}$ concentration at a lower ratio of feed solution to lysate in the reaction than typically used, and a higher $Mg^{2+}$ optimum. These changes essentially eliminate batch-to-batch variability of *L. tarentolae* lysate activity and permit their full potential to be realized.

**KEYWORDS**

cell-free protein expression, in vitro protein translation, Leishmania, *Leishmania tarentolae*, LTE

## 1 | INTRODUCTION

The use of cell-free methods for protein synthesis is now commonplace. A wide variety of systems are available, each with their own strengths and weaknesses. For example, wheat-germ extract, one of the first systems to be developed, is well characterized and has been developed such that protein synthesis by means of this system can rapidly and reliably deliver milligram quantities of protein (Harbers, 2014; Takai & Endo, 2010; Takai, Sawasaki, & Endo, 2010). However, the wheat-germ system is expensive, and preparation of wheat-germ extract is complex and time-consuming. By contrast, *E. coli* cell-free extract is both cheaply and rapidly produced, but does not provide post-translational modifications, and is less successful at producing high-quality eukaryotic proteins, than wheat-germ, or other eukaryote-derived systems, such as rabbit reticulocyte lysate (Gagoski et al., 2016). Nonetheless, because of the ease and low cost of its production, considerable efforts have been made to improve the *E. coli* cell-free system. In the main, these efforts have focussed on the supply of energy to the translation reaction (Calhoun & Swartz, 2007), with glycolytic intermediates (H. C. Kim et al., 2008), glucose, oligosaccharides (H.-C. Kim, Kim, & Kim, 2011; Wang & Zhang, 2009), and combinations of these methods (T. W. Kim et al., 2007) being tested. In addition, a shift toward the use of components which more faithfully mimic the cell cytoplasm (the so called "cytomim" system) has been

  

examined (Calhoun & Swartz, 2007; Jewett & Swartz, 2004). Alternative improvements have been described by Pedersen, Hellberg, Enberg, & Karlsson, 2011, who performed a number of rounds of optimization on a traditional *E. coli* cell-free system. A shift from an S30 to an S12 extract, together with minor concentration changes to various components, increases in particular amino acids (Ser, Gln), and the use of malic acid, 2-oxoglutaric acid and succinic acid as additives being shown to increase yields two to three-fold.

Several other sources for cell-free systems have also been studied, such as those from insect cells (Kubick, Gerrits, Merk, Stiege, & Erdmann, 2009; Merk et al., 2015; Stech et al., 2012), yeast (Gan & Jewett, 2014; Hodgman & Jewett, 2013; Schoborg, Hodgman, Anderson, & Jewett, 2014) and the protozoan lizard parasite *Leishmania tarentolae* (Gagoski et al., 2016; Johnston & Alexandrov, 2014; Kovtun, Mureev, Johnston, & Alexandrov, 2010; Kovtun et al., 2011; Mureev, Kovtun, Nguyen, & Alexandrov, 2009; Ruehrer & Michel, 2013), each with their own advantages and disadvantages. We routinely use the *L. tarentolae* system, since it offers the low cost, ease of culture and simple cell-free lysate production of the *E. coli* system, with the ability to more faithfully express eukaryotic proteins (Gagoski et al., 2016). In our laboratory, this system is used to co-express human proteins of interest for protein-protein interaction studies by AlphaScreen and single molecule fluorescence technologies (Gambin et al., 2014; Han et al., 2014; Sierecki et al., 2013; Sierecki et al., 2014). However, our use of this system has been plagued with difficulties arising from apparent batch-to-batch variations in lysate activity and magnesium dependency. Further, to our knowledge, no comprehensive optimization of the *L. tarentolae* system has been published.

Routine growth of *L. tarentolae* and production of cell-free lysate was easily established, but protein expression by this system had significant lysate batch-to-batch variations in both activity—the overall yield of expressed proteins—and expression quality—the degree of truncated expression, or of aggregation of expressed proteins observed. A related issue was the lysate batch-to-batch variation of the dependence of expression activity on magnesium concentration, as noted in (Johnston & Alexandrov, 2014). Further, the results of test-scale investigations of supplementation conditions and subsequent large-scale supplementations were not consistent, with conditions established in the former often yielding little to no activity when applied to large scales. We therefore undertook a program of optimization of reaction conditions and of the feed solution for cell-free transcription to eliminate this variability and to better understand the *L. tarentolae* cell-free system.

## 2 | MATERIALS AND METHODS

*Leishmania tarentolae* Parrot strain was obtained from Jena Bioscience GmBH, Jena, Germany, as the Lexsy host P10 for constitutive expression. *L. tarentolae* cultures were maintained in modified Terrific broth with both glycerol and glucose (TBGG), containing 0.2% v/v Penicillin/Streptomycin (Life Technologies, Australia Pty. Ltd., Mulgrave, VIC, Australia) and 0.05% w/v Hemin (MP Biomedicals, Santa Ana, CA), in 50 ml volumes (Johnston & Alexandrov, 2014). For lysate production, culture volumes were sequentially increased to 100 ml, 1 L, and 4 L. 4 L cultures were grown in a Biostat A fermentor (Sartorious Stedim, Australia Pty. Ltd., Dandenong South, VIC, Australia) for ~18 hr in TBGG medium with penicillin/streptomycin and hemin additions as above. During the final 4 L growth, culture oxygen tension was maintained at 10% or above, and pH was controlled at 7.4.

### 2.1 | Lysate production

Lysate production was carried out as described in (Johnston & Alexandrov, 2014). Briefly, *L. tarentolae* cells were harvested at an OD of ~6 by centrifugation at 2500*g*. Harvested cells were washed twice by resuspension in ice cold 45 mM HEPES, pH 7.6, containing 250 mM Sucrose, 100 mM Potassium Acetate, and 3 mM Magnesium Acetate (SuEB). Cells were then resuspended to 0.25 g wet weight cells per g cell suspension in SuEB and were placed in a cell disruption vessel (Parr Instruments, Moline, IL). The cell suspension was incubated under 7000 KPa nitrogen for 45 min on ice, then the cells were lysed by rapid release of pressure. The cell lysate was clarified by sequential centrifugation at 10,000*g* and 30,000*g* and anti-splice leader DNA oligonucleotide was added to 10 μM. The lysate was then desalted into 45 mM HEPES, pH 7.6, containing 100 mM Potassium Acetate and 3 mM Magnesium Acetate (EB) using illustra NAP-25 columns, snap-frozen in liquid nitrogen in small aliquots and stored at −80 °C until required.

### 2.2 | Plasmid DNA

Plasmids for expression were constructed using the pCellFree backbone (Gagoski et al., 2015) with insertions cloned from the human ORFeome library described in (Škalamera et al., 2011). Plasmid stocks were prepared by midi-prep (ZymoPURE Midiprep Kit, Zymo Research, Irvine, CA) and normalized to 500 ng DNA/μL. The following plasmids were routinely used: pCellFree_G04-SOX18 (SOX18-GFP), pCellFree_G08-SOX18 (SOX18-Cm), pCellFree_G04-RBPJ (RBPJ-GFP), pCellFree_G03-CAV1 (GFP-CAV1), pCellFree_G08-VAMP2 (VAMP2-Cm), pCellFree_G10-mCherry (mCh-sfGFP).

### 2.3 | Protein expression using *L. tarentolae* lysate

Protein expression was performed in batch reactions. A nominally 5 × concentrated Feeding solution (FS) was used, which contained 8.5 mM ATP, 3.18 mM GTP, 2.5 mM CTP, 2.5 mM UTP, 1.2 mM spermidine, 10 mM DTT, 200 mM creatine phosphate, 100 mM HEPES-KOH pH 7.6, 5% (v/v) PEG 3350, 5 × complete protease inhibitor, 3.5 mM amino acid mixture, 1 mM anti-splice leader DNA oligonucleotide, 5 U/μl creatine phosphokinase. When diluted at a FS: (FS + Lysate) ratio (FSR) of 0.2 (i.e., to a nominal 1 × dilution), this gave 1.7 mM ATP, 0.635 mM GTP, 0.5 mM CTP, 0.5 mM UTP, 0.24 mM spermidine, 2 mM DTT, 40 mM creatine phosphate, 20 mM HEPES-KOH pH 7.6, 1% (v/v) PEG 3350, 1 × complete protease

inhibitor, 0.133 mM amino acid mixture, 0.2 mM anti-splice leader DNA oligonucleotide, 1 U/μl creatine phosphokinase.

In typical expression reactions, thawed *L. tarentolae* lysate was first combined with FS to the desired FSR. T7 DNA polymerase, expressed from the Sigma–Aldrich TargeTron Vector pAR1219, (Sigma–Aldrich Australia Pty. Ltd., Sydney, NSW, Australia) and purified by the method of (Davanloo, Rosenberg, Dunn, & Studier, 1984) RNaseOUT (Life Technologies) and $Mg^{2+}$ (as the acetate salt) were then added to the desired concentrations. Where further dilution of the Lysate-Feed Solution mixture was desired, MilliQ water containing magnesium acetate was used, such that the $Mg^{2+}$ concentration remained constant. Expression reactions were started by the addition of 9 μL aliquots of this master mix to 1 μL (500 ng) of plasmid DNA. Expression reactions were performed in 200 μL PCR tube strips, at 27 °C, for 3 hr. Tube caps were pierced to permit the maturation and full development of mCherry fluorescence. Variations to these conditions are noted in the text.

Protein expression was quantitated by means of GFP or mCherry fluorescence. Fluorescence was measured in real time using a Synergy 4 plate reader (BioTek, Winooski, VT), or at discrete time points using a Chemidoc MP imaging system (Bio-Rad, Laboratories Pty. Ltd., Gladesville, NSW, Australia) equipped with epi-red, epi-green, and epi-blue LED modules. Protein expression was also quantitated by SDS-PAGE, with detection of expressed GFP- or mCherry-tagged constructs by means of the Chemidoc MP imaging system. Samples for gels were prepared by combining 10 μL sample aliquots with 4 μL Bolt LDS sample buffer (Life Technologies Australia Pty. Ltd.). A total of 4 μL samples were then loaded onto precast Novex BOLT 4–12% Bis Tris gels (Life Technologies Australia Pty. Ltd.). Following electrophoresis, gels were imaged without further processing, using the inbuilt Alexa 488 (GFP), Alexa 546 (mCherry), and Cy5 (prestained markers) settings.

## 3 | RESULTS

To understand the potential origins of batch-to-batch variability, we set out to systematically explore the effect of the different parameters on protein expression. This was assessed by quantifying the fluorescence of GFP or mCherry-tagged proteins under different expression conditions. We started with the most obvious parameters, such as temperature, magnesium concentration, pH of reactions, before a chance observation lead us to vary more parameters.

Initial experiments established a broad temperature optimum between 21 and 27 °C, the exact optimum depending on the construct used (Supplementary Figure S1a–f). Expression temperature had little effect on the quality of protein expression, as, determined by SDS-PAGE, with the exception of the SOX18-GFP construct (Supplementary Figure S1g–j). SOX18-GFP expression reactions produced a large number of lower mass species, notably at ~30 kDa, ~50 kDa, and multiple bands between 55 kDa and 70 kDa. The lower mass bands followed a similar distribution with temperature to the overall expression levels, however the multiple bands over 55 kDa were suppressed above 29 °C, and the full length SOX18-GFP, essentially absent below 18.8 °C, became more prominent. For instrument-related reasons, expressions were routinely carried out at 27 °C. Because of its sensitivity to expression conditions, SOX18-GFP was routinely used in testing, together with the mCh-sfGFP tandem construct. This allowed full length expression to be tracked, together with folding—sfGFP is a highly robust, fast folder, while mCherry folds, and matures slowly (Pedelacq, Cabantous, Tran, Terwilliger, & Waldo, 2006; Shaner et al., 2004).

### 3.1 | Effect of magnesium concentration

Magnesium ion concentration is well known to be a critical parameter in cell-free expression, since it acts as a counter-ion to the nucleotide phosphates used both as energy sources for the expression and as raw materials for the transcription portion of expression. In order to assess the role of magnesium in protein expression by *L. tarentolae* lysate, the magnesium dependence of expression of SOX18-GFP and mCh-sfGFP was investigated over a wide range of magnesium concentrations (2–32 mM), in a plate reader, so that effects on expression kinetics could also be assessed. Figure 1a shows that despite the optimal concentration for expression for the three lysates tested being similar (4–6 mM), detectable activity remained at concentrations up to 20 mM $Mg^{2+}$. Inspection of the time courses (Figure 1b), and rates of expression (Figure 1c) suggested that for two of the lysates at $[Mg^{2+}]$ greater than optimum, in addition to decreased overall expression, the time taken to reach maximal expression and the lag time for commencement of the reaction both increased. In the third lysate, the maximum rate of expression was reached at a significantly higher $[Mg^{2+}]$, and the time taken to reach maximum rate, and the lag time decreased as $[Mg^{2+}]$ increased. Subtraction of the lag time from the time taken to maximum rate (Figure 1d) removed most of the effect of changing $[Mg^{2+}]$, suggesting that the changes observed in the expression kinetics were due to changes in the lag times of the expression reaction. Analysis of the expression products by SDS-PAGE demonstrated no significant differences in expression quality over the range of magnesium concentration investigated (not shown).

### 3.2 | Effect of feed solution ratio

During one of the screening phases, reactions were set up with smaller volumes, and 1 μL of pure water was omitted from the 10 μL total volume of the mix. To our surprise, this small variation led to a complete shutdown of protein expression in the system. Repeats of the same experiments showed that the balance between lysate and feed solution was critical.

We set up to investigate in more detail the importance of the feed solution ratio, here defined as the ratio of the volume of feed solution to volume of total reaction, that is,

$$\mathrm{FSR} = \frac{\mathrm{Vol.FS}}{(\mathrm{Vol.FS} + \mathrm{Vol.Lysate})}$$

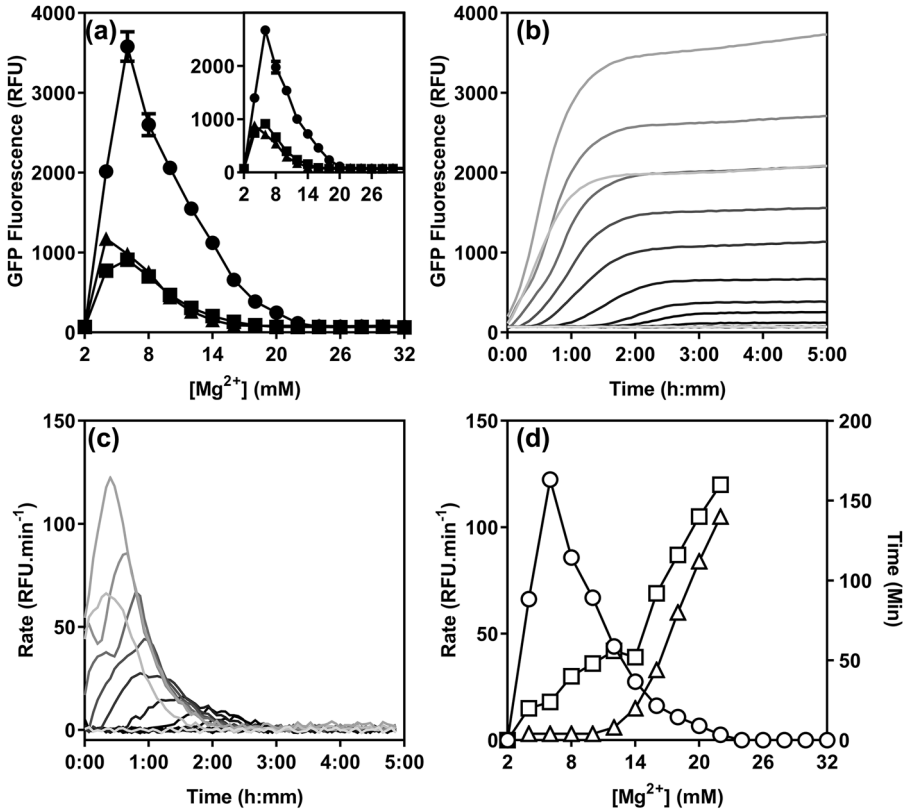


**FIGURE 1** shows the effect of varying the magnesium concentration on expression by *L. tarentolae* cell-free lysate. (a) total expression of SOX18-GFP by three separate lysates (1: solid circles; 2: solid squares; 3: solid triangles) at varying concentrations of magnesium. Inset, total expression of mCh-sfGFP by the same lysates, measured by GFP fluorescence. (b) Expression of SOX18-GFP with time for lysate 1, at increasing concentrations of magnesium from 2 mM (lightest gray) to 32 mM (black). Data for mCh-sfGFP was similar. (c) Changes in the rate of SOX18-GFP expression (i.e., increase in fluorescence/minute − the slopes of graphs in b) with time, at increasing concentrations of magnesium from 2 mM (lightest gray) to 32 mM (black). (d) Maximum rate of expression (open circles), time to reach maximum rate (open squares) and the reaction lag time (open triangles) defined as the time for the reaction rate to exceed 5 RFU.min$^{-1}$. Subtraction of the lag time from the time to reach maximum rate indicated that the changes in kinetics seen in b and c were predominantly due to changes in the lag time

The normal reaction set up (Johnston & Alexandrov, 2014) uses an FSR of 0.2; varying this ratio alters the concentration of feed solution components to lysate in a concerted manner.

Figure 2a shows expression of SOX18-GFP and mCh-sfGFP at four FSR values, each at three concentrations of magnesium. This reveals that at 5 mM $Mg^{2+}$, expression of SOX18-GFP drops by ~65% when the FSR is altered from 0.18 to 0.21. In a 10 µL reaction, this corresponds to a change in added feed solution of 0.3 µL, well within pipetting error, especially of the somewhat viscous feed solution. Over the FSR range 0.15–0.24, corresponding to a volume change of 0.9 µL, the expression activity falls by approximately 90%. Though such volume errors can be mitigated by using larger volume master mixes, such major changes in activity with minor changes in reaction composition are noteworthy.

Following averaging within each FSR value, comparison of the standard deviations and coefficients of variation between each suggests that lysate activity is more stable to variations in the concentration of magnesium at FSR values below 0.2 than at values above 0.2 (Figure 2b). A survey of nine individual *Leishmania* lysates (Supplementary Figures S2a and S2b) revealed that this instability of expression activity at FSR values greater than 0.2 with respect to the concentration of magnesium, was generally observed.

The range of FSR values was then extended, to encompass a range of 0.1 (1 µL FS per 9 µL lysate) to 0.3 (3 µL FS per 7 µL lysate). The consequent range of concentrations of feed solution components is given in Supplementary Table S1. Analysis of four individual *Leishmania* lysates over this range confirmed the initial observation, and suggested that, for optimal activity, FSR values below 0.2 should be used (Figures 3a and 3b).

## 3.3 | Effect of feed solution components

In order to investigate which of the feed solution components, varied in concert in the FSR experiments, was likely to be responsible for the effects observed, the effects of varying each individually was investigated. Feed solutions were made lacking each component in turn. These were then supplemented with the component under investigation over a range of final concentrations from 0 to 3 times that under standard (FSR = 0.2) conditions. All other components were maintained at the standard concentrations except for PEG 3350, which was reduced from nominally 1% to 0.25% on the basis of preliminary

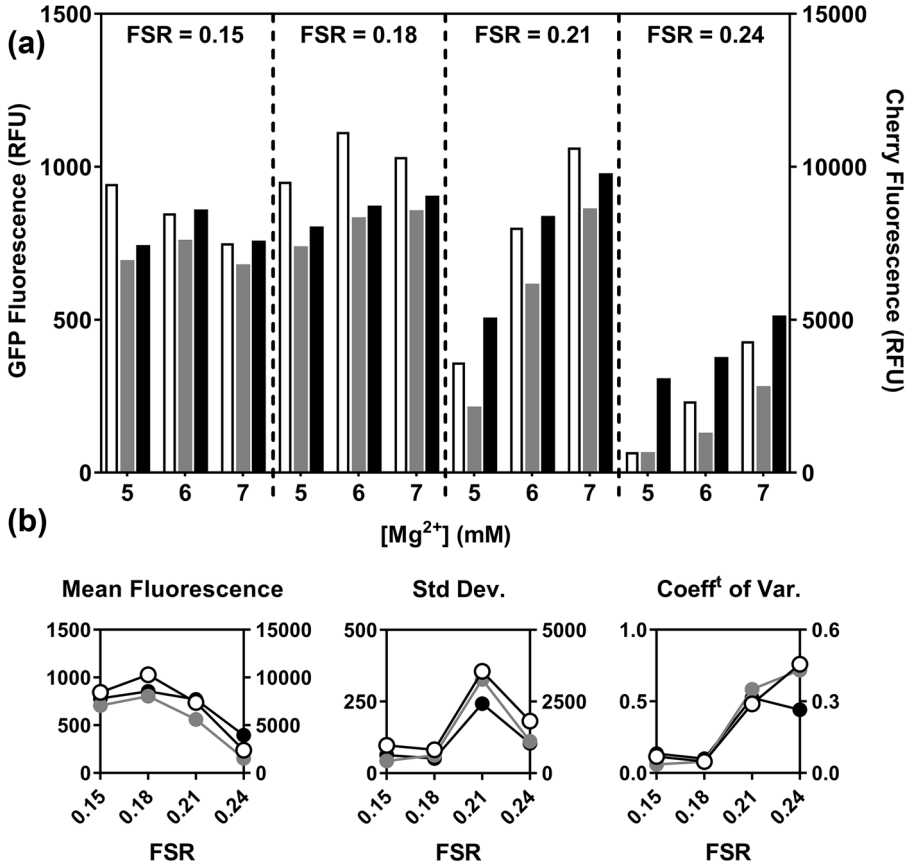


**FIGURE 2** Panel (a) shows the expression levels obtained at four separate FSR values from 0.15 to 0.24, each at three magnesium concentrations. Inspection of the resulting bar graph suggests that the variation within a FSR set with respect to magnesium concentration is greater for the two higher values (0.21, 0.24). Panel (b) shows this effect quantitated, with higher standard deviations and coefficients of variation obtained for these higher FSR values when the expression levels are averaged over the three magnesium concentration within a FSR value. SOX18-GFP: Open bars/circles; mCh-sfGFP quantitated using GFP: gray solid bars/circles; quantitated using mCherry: black solid bars/circles

experiments. The previously determined optimum FSR was used. Figure 4a–i summarizes the results obtained. For simplicity, CTP and UTP concentrations were varied together, and the concentrations of protease inhibitor cocktail, antisense oligonucleotide, and T7 polymerase were maintained constant throughout.

Varying the reaction pH had only minor effects on protein expression (Supplementary Figures S3a and S3b). Other components fell into three broad categories: 1. dithiothreitol (DTT), PEG 3350, which had little effect on expression at any concentration tried; 2. ATP, GTP, CTP/UTP, amino acids, creatine phosphokinase, which produced little or no expression when absent, but which increased expression to a maximum as their concentrations increased, with little change in expression above this concentration; and 3. creatine phosphate, spermidine, which had a clear optimum concentration, above and below which expression was reduced. As seen previously, none of the treatments appeared to alter the quality of the expressed protein, merely the amount of expression, as judged by SDS-PAGE (data not shown). The most important observation was that the expression activity changes resulting from concentration changes in each individual component which would be caused by varying the FSR of the range 0.1–0.3 (shaded blocks) was insufficient to fully explain the often dramatic changes in overall protein expression activity observed.

These experiments had, however, neglected the effect of magnesium on the expression reactions. Therefore, a subset of components were re-examined over the same 0–3 times standard concentrations, at final magnesium concentrations from 4 mM to 14 mM. The data obtained are summarized in Figure 5a–f. At low concentrations of magnesium, ATP shows a narrow peak of expression activity with concentration at low ATP concentrations, which markedly broadens and shifts to a higher ATP concentration as the magnesium concentration increases. GTP also shows a peak of expression activity at low GTP concentrations when the magnesium concentration is low. This shifts to higher GTP concentration as the concentration of magnesium increases. However, further increase in the magnesium concentration reduces the overall expression activity, which falls close to 0 at 14 mM magnesium.

Creatine phosphate also shows a narrow peak of expression activity at low concentration when the concentration of magnesium is low. Thus at 4 mM $Mg^{2+}$, an increase in creatine phosphate concentration from 34.3 mM (the approximate equivalent of a FSR of 0.16) to 51.4 mM (the approximate equivalent of a FSR of 0.26) essentially abolishes activity. The peak of activity shifts to slightly higher creatine phosphate concentrations as the magnesium concentration increases, but overall the expression activity decreases.

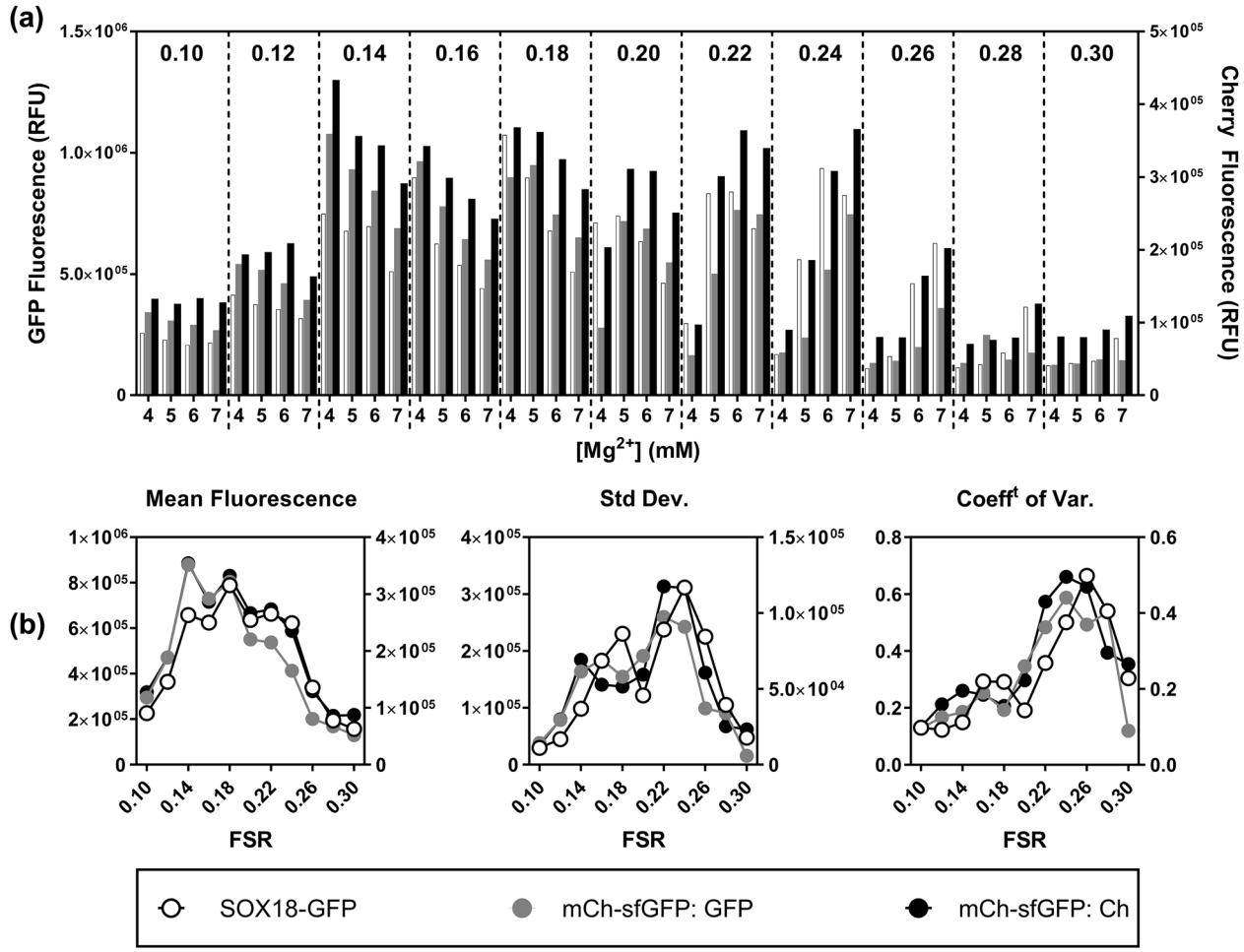


**FIGURE 3** shows a similar experiment to that displayed in Figure 2, extending over a wider range of FSR and magnesium concentration. Though the variation is less apparent on inspection of the bar graph, a similar pattern of standard deviations and coefficients of variation to those seen in Figure 2 panel (b) are observed

Interestingly, the expression activity at the highest concentrations of creatine phosphate increases markedly at 14 mM magnesium.

Both DTT and amino acids show a simple suppression of expression activity as the concentration of magnesium increases, while the narrow optimum concentration peak for spermidine shifts to a lower concentration and is then suppressed as the magnesium concentration increases.

## 3.4 | Pre-incubation of lysate with feed solution

Pre-incubation experiments to test the stability of the protein expression activity of lysate may offer clues as to where to direct optimisation efforts, by indicating which reaction components are degraded during expression (D. M. Kim & Swartz, 2000). *Leishmania* lysate lost essentially all activity when incubated alone, on ice. When incubated in the presence of feed solution, or in the presence of the complete reaction mix (i.e., feed solution, T7 polymerase, $Mg^{2+}$, RNaseOUT), retention of expression activity was observed at up to 2 hr, albeit at low levels; ~20% at 2 hr when pre-incubated with the complete reaction mix (Supplementary Figure S3a–c). Attempts were made to improve retention of activity by the use of common protein stabilization reagents. Glycerol, commonly used to stabilize proteins, was shown to inhibit expression activity in the *L. tarentolae* system (data not shown). Alternative stabilizers; sucrose, mannose, trehalose, and sorbitol, did not inhibit the lysate activity, and had at best a minor protective effect on pre-incubation lysate stability above that seen with complete reaction mix (Supplementary Figure S4d–f).

The phosphatase inhibitor PhosSTOP (Sigma–Aldrich) was also investigated, since prevention of premature hydrolysis of nucleotide phosphates and creatine phosphate by endogenous phosphatases might be expected to increase their availability for use in the transcription/translation reactions in lysate. However, inclusion of PhosSTOP in concentrations as low as 0.1 × the recommended concentration essentially eliminated protein expression activity (data not shown).

## 3.5 | Feeding of expression reactions and effect of phosphate

It was noted that in most reactions, protein expression ceased ~2 hr after beginning; see for example, Figures 1b and 1c. Attempts were

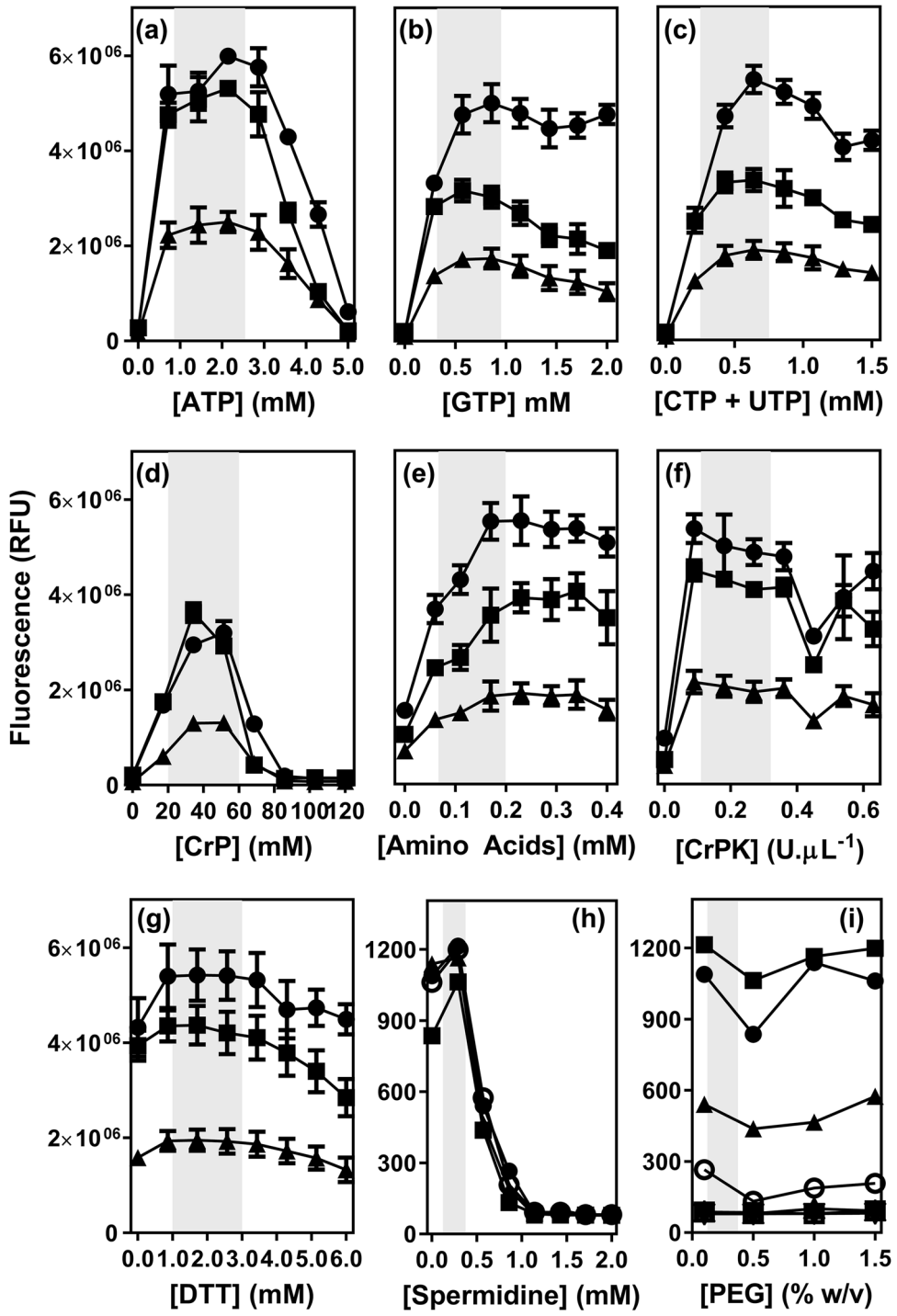


**FIGURE 4** (a–g) Lysate activity versus feed solution component concentration, using SOX18-GFP (solid circles) and mCh-sfGFP, (GFP expression: solid squares; mCh expression: solid triangles) as test DNAs. Expression was quantified by Chemidoc. (h) Lysate activity versus spermidine concentration, at four concentrations of PEG 3350, using SOX18-GFP as test DNA—mCh-sfGFP data was similar. Solid circles: 0.1% (v/v) PEG; solid squares: 0.5% PEG; solid triangles: 1.0% PEG; open circles: 1.5% PEG. I: data from h plotted as lysate activity versus PEG 3350 concentration. Solid circles: 0.00 mM Spermidine; solid squares: 0.29 mM spermidine; solid triangles: 0.57 mM spermidine; open circles: 0.86 mM spermidine; open squares: 1.14 mM spermidine; open triangles: 1.43 mM spermidine; solid down triangles: 1.71 mM spermidine; open down triangles: 2.00 mM spermidine. In this case, expression was quantitated by plate reader. In all panels, the shaded area indicates the range of component concentration in the final reaction produced by varying FSR values between 0.1 and 0.3

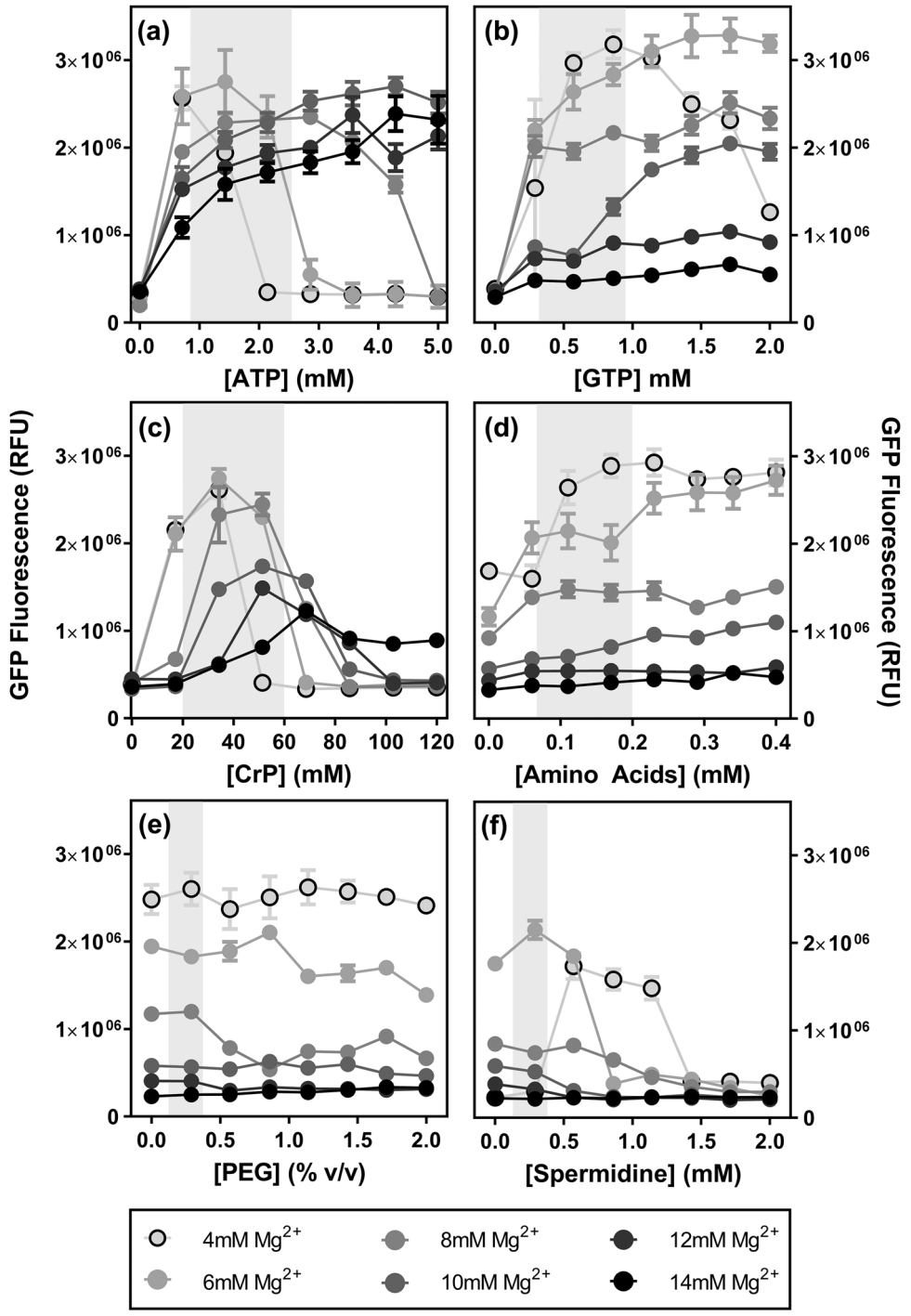


**FIGURE 5** Lysate activity versus feed solution component concentration, using mCh-sfGFP as test DNA, at six magnesium concentrations from 4 mM (light gray, open circles) to 14 mM (black, solid circles). Expression was quantitated by Chemidoc, using GFP fluorescence; mCherry fluorescence was similar. In all panels, the shaded area indicates the range of component concentration in the final reaction produced by varying FSR values between 0.1 and 0.3

therefore made to prolong the expression reaction by feeding with either feed solution, amino acids, or additional $Mg^{2+}$. No extension of the expression reaction was observed; in all cases where additions were made, the expression was reduced compared to expression where no additions were made (Supplementary Figure S5a–f).

Since these results suggested that depletion of reaction components was not the cause of expression ceasing, attention was turned to the build-up of waste products, principally inorganic phosphate, which is known to affect cell-free expression. The effect of inorganic phosphate on protein expression was tested, and a marked inhibition of protein expression was observed above 20 mM, with complete inhibition of expression by 60 mM added potassium phosphate (Supplementary Figures S5a and S5b). It was also noted that though the magnitude of protein expression was dramatically affected, additional phosphate did not appear to affect the

kinetics of the reaction—neither the time taken for the reaction to reach maximum rate, or for the reaction to cease was affected (Supplementary Figure S6c–f).

## 4 | DISCUSSION

Cell-free lysate systems for the expression of proteins are clearly complex, with requirements for many additional components, each of which can have a dramatic effect on the overall activity of the lysate, and on the quality of the material produced. It is therefore necessary to conduct comprehensive testing on the cell-free system in order to obtain the best performance for the desired task. This performance may be a trade-off between quantity of expressed material and quality, since changes to the system to maximize quantity may result in an increase in errors in protein expression, truncations, and the like. This study examines the performance of the *Leishmania tarentolae* cell-free protein expression system in detail, in order to provide the basis for optimization of expression, and to solve issues with instability of the system activity with respect to additional components of the reaction.

Initial experiments with this system indicated that the *L. tarentolae* lysate behaved as published, with magnesium and pH dependencies broadly matching those obtained previously (Johnston & Alexandrov, 2014). The temperature optimum for the majority of constructs investigated was found to be lower than the 27 °C specified in this protocol, with the exception of the SOX18-GFP construct. In this case, a higher temperature was beneficial in producing full-length protein, but this had to be balanced against the lower expression yield at temperatures above 30 °C. While 27 °C may be an acceptable compromise temperature for general protein expression, it is clear that in specific cases, optimization of the expression temperature may be necessary to obtain the expression yield and protein quality desired.

The lability of *L. tarentolae* lysate noted in (Johnston & Alexandrov, 2014) was confirmed by pre-incubation experiments. Though a measure of protection of the expression activity of the lysate was obtained by supplementation with feed solution, this was relatively small. Attempts to further stabilize the lysate using common reagents—glycerol, sugars, phosphatase inhibitor—were unsuccessful. Similarly, feeding experiments designed to alleviate reaction component or magnesium depletion were unsuccessful, suggesting that the lability of the lysate is the main cause of loss of activity. This is supported by the effect of additional phosphate, which decreases the overall yield, but which does not appear to have any effect on the kinetics of the reaction, suggesting a separate mechanism may operate to cause cessation of protein expression.

In our hands, the *L. tarentolae* expression system displayed an expression reaction-to-expression reaction instability, which manifested as a marked sensitivity to magnesium concentration over and above the expected lysate-to-lysate variation, when used as described by Johnston and Alexandrov (2014), at 2 volumes feed solution to 8 volumes lysate (FSR = 0.2). A chance observation that altering the ratio of feed solution to lysate resulted in much less sensitivity to magnesium concentration, led to a systematic investigation of the effects of varying the feed solution ratio and of the reasons for the stability/instability discovered. Analysis of several individual lysate batches (Supplementary Figures S2a and S2b) suggested that, in the main (lysates B, C, D, and F), protein expression activities showed a "stable" region with respect to magnesium concentration at FSR values below 0.2, while at FSR = 0.2 and above, protein expression activity was much less stable with respect to the magnesium concentration, that is, changes in the magnesium concentration were more likely to result in large changes in expression activity. The other lysates (A, E, G, H, I) showed a peak of instability at FSR = 0.18 – 0.21, depending on the construct used, with greater stability to magnesium at the extremes of FSR investigated. However, the apparent high stability at high FSR values observed is likely an artifact of the low to negligible activities at these values, indicating that despite the decreased stability to changes in magnesium concentration, expressing at a low FSR is preferable

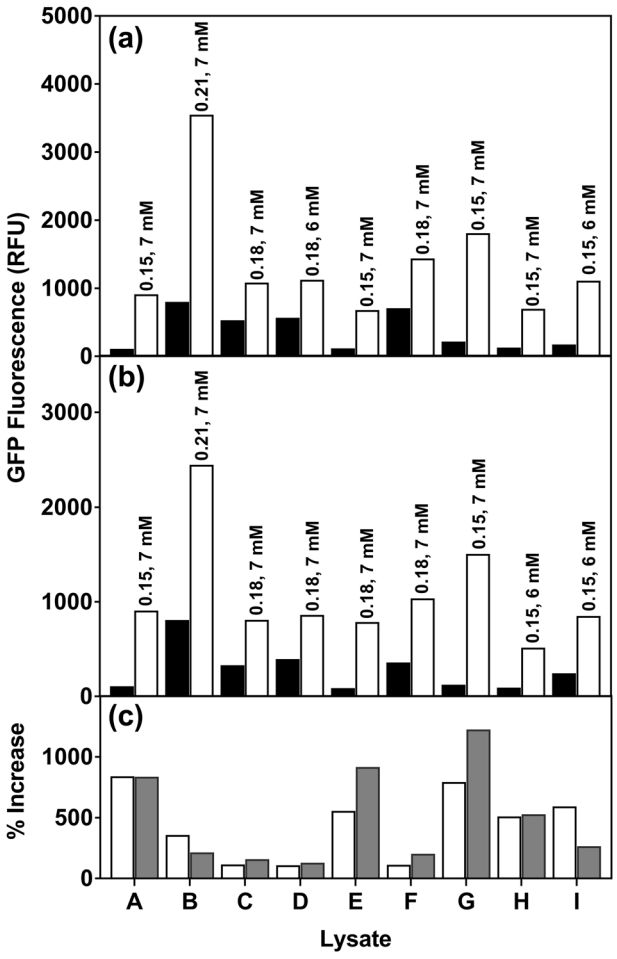


**FIGURE 6** Comparison of expression yield from nine lysates under "typical" conditions ((Johnston & Alexandrov, 2014): 5 mM $Mg^{2+}$, FSR = 0.2–values interpolated from FSR = 0.18 and FSR = 0.21 conditions, black bars) and under the best conditions obtained in our experiments (open bars). Magnesium concentrations and FSR values producing the maximum expression activity are given above the bars for the corresponding lysates. Panel A: SOX18-GFP expression; Panel B: mCh-sfGFP expression; Panel C: percentage improvement in expression on changing the expression condition from "typical" to best (SOX18-GFP: open bars; mCh-sf-GFP, gray bars)

The initial observations of the effects of varying individual feed solution components did not yield an explanation for the effects of varying the feed solution ratio, since the range of component concentrations accessed by varying the FSR between 0.1 and 0.3 (Figure 4a–i, shaded areas) did not appear to be sufficient to account for the large changes in expression activity seen. Only when the effect of the reaction magnesium concentration was simultaneously investigated, did changes of sufficient magnitude to be responsible for the observed effects of varying FSR on the expression activity become apparent (Figure 5a–f). Given the typical reaction volume of 10–20 μL, and a typical stock solution of 100 mM magnesium, a minor error in pipetting of 0.1 μL would be sufficient to change the final magnesium concentration in an expression reaction by 1 mM. This would be of minor consequence in the "stable" region of feed solution ratio. However, in the "unstable" region of feed solution ratio, typically at and above 0.2, it is sufficient to reduce expression activity dramatically (compare the results of a 1 mM change in magnesium concentration at FSR 0.18 and at 0.21, Figure 2). In this region, minor errors in pipetting may lead to a "low-quality lysate."

As a result of these findings we now routinely screen new lysates at a range of FSR values and magnesium concentrations, as shown in Figures 2, S2a, S2b. Inspection of these results then allows the determination of the best ratio of lysate to feed solution to maximize stability of the expression reaction to magnesium concentration. Figure 6 shows the increase in activity obtained by this screening, as well as the conditions which yield maximum expression, in comparison to the "typical" conditions (Johnston & Alexandrov, 2014) (FSR = 0.2, 5 mM $Mg^{2+}$). It is then possible to balance the desire for stability to magnesium concentration against that for the greatest expression activity. For example, lysate B produces maximum activities at FSR = 0.21, 7 mM $Mg^{2+}$. However, it may be preferable to accept a minor loss in activity and obtain the greater stability to magnesium concentration offered by FSR = 0.18 (Supplementary Figures S2a and S2b). Lysates may thus be produced which perform to their maximum potential for the desired application.

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## REFERENCES

Calhoun, K. A., & Swartz, J. R., (2007). Energy systems for ATP regeneration in cell-free protein synthesis reactions. In G. Grandi, (Ed.), *In Vitro Transcription and Translation Protocols*. 2007/07/20 ed., Vol. 375 (pp. 3–17). Totowa, NJ: Humana Press, Inc.

Davanloo, P., Rosenberg, A. H., Dunn, J. J., & Studier, F. W. (1984). Cloning and expression of the gene for bacteriophage T7 RNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(7), 2035–2039.

Gagoski, D., Mureev, S., Giles, N., Johnston, W., Dahmer-Heath, M., Skalamera, D., . . . Alexandrov, K. (2015). Gateway-compatible vectors for high-throughput protein expression in pro- and eukaryotic cell-free systems. *Journal of Biotechnology*, *195*, 1–7. https://doi.org/10.1016/j.jbiotec.2014.12.006.

Gagoski, D., Polinkovsky, M. E., Mureev, S., Kunert, A., Johnston, W., Gambin, Y., & Alexandrov, K. (2016). Performance Benchmarking of Four Cell-Free Protein Expression Systems. *Biotechnology and Bioengineering*, *113*(2), 292–300. https://doi.org/10.1002/bit.25814.

Gambin, Y., Ariotti, N., McMahon, K. A., Bastiani, M., Sierecki, E., Kovtun, O., . . . Parton, R. G. (2014). Single-molecule analysis reveals self assembly and nanoscale segregation of two distinct cavin subcomplexes on caveolae. *Elife*, *3*, 18. https://doi.org/10.7554/eLife.01434.

Gan, R., & Jewett, M. C. (2014). A combined cell-free transcription-translation system from Saccharomyces cerevisiae for rapid and robust protein synthesis. *Biotechnology Journal*, *9*(5), 641–651. https://doi.org/10.1002/biot.201300545.

Han, S. P., Gambin, Y., Gomez, G. A., Verma, S., Giles, N., Michael, M., . . . Yap, A. S. (2014). Cortactin scaffolds arp2/3 and WAVE2 at the epithelial zonula adherens. *Journal of Biological Chemistry*, *289*(11), 7764–7775. https://doi.org/10.1074/jbc. M113. 544478.

Harbers, M. (2014). Wheat germ systems for cell-free protein expression. *FEBS Letters*, *588*(17), 2762–2773. https://doi.org/10.1016/j.febslet.2014.05.061.

Hodgman, C. E., & Jewett, M. C. (2013). Optimized extract preparation methods and reaction conditions for improved yeast cell-Free protein synthesis. *Biotechnology and Bioengineering*, *110*(10), 2643–2654. https://doi.org/10.1002/bit.24942.

Jewett, M. C., & Swartz, J. R. (2004). Mimicking the Escherichia coli cytoplasmic environment activates long-lived and efficient cell-free protein synthesis. *Biotechnology and Bioengineering*, *86*(1), 19–26. https://doi.org/10.1002/bit.20026.

Johnston, W. A., & Alexandrov, K., (2014). Production of eukaryotic cell-free lysate from Leishmania tarentolae. In K. Alexandrov, & W. A. Johnston, (Eds.), *Cell-Free protein synthesis: Methods and protocols*. Vol. 1118 (pp. 1–15). Totowa, NJ: Humana Press, Inc.

Kim, D. M., & Swartz, J. R. (2000). Prolonging cell-free protein synthesis by selective reagent additions. *Biotechnology Progress*, *16*(3), 385–390. https://doi.org/10.1021/bp000031y.

Kim, H.-C., Kim, T.-W., & Kim, D.-M. (2011). Prolonged production of proteins in a cell-free protein synthesis system using polymeric carbohydrates as an energy source. *Process Biochemistry*, *46*(6), 1366–1369. https://doi.org/10.1016/j.procbio.2011.03.008.

Kim, H. C., Kim, T. W., Park, C. G., Oh, I. S., Park, K., & Kim, D. M. (2008). Continuous cell-free protein synthesis using glycolytic intermediates as energy sources. *Journal of Microbiology and Biotechnology*, *18*(5), 885–888.

Kim, T. W., Oh, I. S., Keum, J. W., Kwon, Y. C., Byun, J. Y., Lee, K. H., . . . Kim, D. M. (2007). Prolonged cell-free protein synthesis using dual energy sources: Combined use of creatine phosphate and glucose for the efficient supply of ATP and retarded accumulation of phosphate. *Biotechnology and Bioengineering*, *97*(6), 1510–1515. https://doi.org/10.1002/bit.21337.

Kovtun, O., Mureev, S., Johnston, W., & Alexandrov, K. (2010). Towards the construction of expressed proteomes using a Leishmania tarentolae based cell-free expression system. *PLoS ONE*, *5*(12), e14388. https://doi.org/10.1371/journal.pone.0014388.

Kovtun, O., Mureev, S., Jung, W., Kubala, M. H., Johnston, W., & Alexandrov, K. (2011). Leishmania cell-free protein expression system. *Methods*, *55*(1), 58–64. https://doi.org/10.1016/j.ymeth.2011.06.006.

Kubick, S., Gerrits, M., Merk, H., Stiege, W., & Erdmann, V. A., (2009). In Vitro Synthesis of Posttranslationally Modified Membrane Proteins. In L. DeLucas, (Ed.), *Current topics in membranes*. Vol. 63 (pp. 25–49). Cambridge, MA: Academic Press.

Merk, H., Rues, R. B., Gless, C., Beyer, K., Dong, F., Dotsch, V., . . . Bernhard, F. (2015). Biosynthesis of membrane dependent proteins in insect cell lysates: Identification of limiting parameters for folding and processing. *Biological Chemistry*, *396*(9-10), 1097–1107. https://doi.org/10.1515/hsz-2015-0105.

Mureev, S., Kovtun, O., Nguyen, U. T., & Alexandrov, K. (2009). Species-independent translational leaders facilitate cell-free expression. *Nature Biotechnology*, *27*(8), 747–752. https://doi.org/10.1038/nbt.1556.

Pedelacq, J. D., Cabantous, S., Tran, T., Terwilliger, T. C., & Waldo, G. S. (2006). Engineering and characterization of a superfolder green fluorescent protein. *Nature Biotechnology*, *24*(1), 79–88. https://doi.org/10.1038/nbt1172.

Pedersen, A., Hellberg, K., Enberg, J., & Karlsson, B. G. (2011). Rational improvement of cell-free protein synthesis. *New Biotechnology*, *28*(3), 218–224. https://doi.org/10.1016/j.nbt.2010.06.015.

Ruehrer, S., & Michel, H. (2013). Exploiting Leishmania tarentolae cell-free extracts for the synthesis of human solute carriers. *Molecular Membrane Biology*, *30*(4), 288–302. https://doi.org/10.3109/09687688.2013.807362.

Schoborg, J. A., Hodgman, C. E., Anderson, M. J., & Jewett, M. C. (2014). Substrate replenishment and byproduct removal improve yeast cell-free protein synthesis. *Biotechnology Journal*, *9*(5), 630–640. https://doi.org/10.1002/biot.201300383.

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., & Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nature Biotechnology*, *22*(12), 1567–1572. https://doi.org/10.1038/nbt1037.

Sierecki, E., Giles, N., Polinkovsky, M., Moustaqil, M., Alexandrov, K., & Gambin, Y. (2013). A cell-free approach to accelerate the study of protein-protein interactions in vitro. *Interface Focus*, *3*(5), 20130018. https://doi.org/10.1098/rsfs.2013.0018.

Sierecki, E., Stevers, L. M., Giles, N., Polinkovsky, M. E., Moustaqil, M., Mureev, S., . . . Gambin, Y. (2014). Rapid mapping of interactions between human SNX-BAR proteins measured In vitro by AlphaScreen and single-molecule spectroscopy. *Molecular and Cellular Proteomics*, *13*(9), 2233–2245. https://doi.org/10.1074/mc p.M113. 037275.

Škalamera, D., Ranall, M. V., Wilson, B. M., Leo, P., Purdon, A. S., Hyde, C., . . . Gonda, T. J. (2011). A high-Throughput platform for lentiviral over-expression screening of the human ORFeome. *PLoS ONE*, *6*(5), e20057. https://doi.org/10.1371/journal.pone.0020057.

Stech, M., Merk, H., Schenk, J. A., Stocklein, W. F., Wustenhagen, D. A., Micheel, B., . . . Kubick, S. (2012). Production of functional antibody fragments in a vesicle-based eukaryotic cell-free translation system. *Journal of Biotechnology*, *164*(2), 220–231. https://doi.org/10.1016/j.jbiotec.2012.08.020.

Takai, K., & Endo, Y., (2010). The cell-free protein synthesis system from wheat germ. In Y. Endo, K. Takai, & T. Ueda, (Eds.), *Cell-Free protein production: Methods and protocols*. Vol. 607 (pp. 23–30). Totowa, NJ: Humana Press, Inc.

Takai, K., Sawasaki, T., & Endo, Y. (2010). Practical cell-free protein synthesis system using purified wheat embryos. *Nature Protocols*, *5*(2), 227–238. https://doi.org/10.1038/nprot.2009.207.

Wang, Y., & Zhang, Y. H. (2009). Cell-free protein synthesis energized by slowly-metabolized maltodextrin. *BMC Biotechnology*, *9*, 58. https://doi.org/10.1186/1472-6750-9-58.

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