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A validated sfGFP fluorescence assay to monitor Escherichia coli extract productivity for cell- free biomanufacturing

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Abstract

Cell-free protein synthesis represents a rapidly developing class of manufacturing technologies reported to enable rapid, scalable, decentralized, and versatile protein production for drug products. However, the batch-to-batch variability of the cell extract raw material used for the cell-free reaction may affect the production consistency and product quality. Therefore, we developed and validated an *in vitro* superfolder green fluorescent protein (sfGFP) productivity assay to quantify and compare the protein-synthesis output of *Escherichia coli (E. coli)*–derived cell extract batches for suitability prior to their use in establishing a cell-free manufacturing platform. Here, we report a validated quantitative fluorometric assay that is fit-for-purpose for monitoring batch-to-batch variation in extract productivity. This method was evaluated following International Council for Harmonisation (ICH) Q2(R2) and Q14 guidelines to support reproducibility and comparability across laboratories. Assay performance was assessed for specificity, linearity, limit of detection, limit of quantitation, accuracy, robustness, and precision. The assay exhibited a working range of (6.25 to 200) mg/L for sfGFP produced by the cell free reaction with linearity ($R^2 \geq 0.99$) and intermediate precision (< 25% relative standard deviation). To demonstrate the practical application of this method across different laboratories, the assay was applied to monitor extract performance during process scale-up by comparing 1 L laboratory-scale batches produced at FDA with 50 L pilot-scale batches produced at DEVCOM-CBC. To support analytical comparability and standardization efforts across the cell-free manufacturing community, all data and methodological protocols from this study have been made available to academic, institutional, and commercial cell-free manufacturing stakeholders at no cost. These data support the utility of this *in vitro* sfGFP assay to characterize extract productivity and to inform strategies for monitoring extract performance in *E. coli* cell-free manufacturing workflows.

Introduction

Cell-free (CF) manufacturing platforms represent a rapidly developing class of manufacturing technologies reported to enable rapid and decentralized production of various protein biologics [1, 2]. Furthermore, these platform technologies may be portable for fast point-of-need deployment to support public health emergencies and frontline warfighters. CF protein synthesis (CFPS) is most commonly reliant upon crude cell lysates (extracts) as a key raw material containing the transcription and translation biomachinery required to synthesize proteins in an open reaction format [3, 4]. Although this open format enables direct control of reaction conditions, it also increases dependence on the quality and consistency of biological raw materials, particularly the cell extracts [4, 5]. A potential barrier to broader implementation of CFPS is batch-to-batch variability in the cell extracts [5, 6]. Differences in extract sources, fermentation conditions, lysis parameters, synthesis buffer or supplements, and upstream processing may produce substantial changes in extract protein-synthesis capacity [4–8]. A functional readout of translational capacity is often more informative than total protein concentration, which may not reliably predict extract performance. Recognizing these bottlenecks, recent roadmaps, workshops, and reports from the National Institute of Standards and Technology (NIST) have called for the development of standardized analytical measurements and validated quality control assays to

ensure the reproducibility and scalability of CF manufacturing [9–11]. Standardized and validated analytical methods are essential to facilitate characterization and control of extracts and support data-driven approaches to raw-material monitoring [12].

A functional measure of an extract's translational capacity, rather than total protein concentration alone, is particularly important in this context, as the protein translational capability provides a direct and informative readout of extract performance. In this study, we developed a functional assay to measure the translational capacity of the extract that follows guidelines established by the International Council for Harmonisation (ICH), an international body that develops harmonized technical standards for pharmaceutical development and regulation. Specifically, ICH Q2(R2) provides a structured framework for the validation of analytical procedures, defining the performance characteristics, such as specificity, linearity, accuracy, and precision, that must be demonstrated to confirm a method is fit for its intended purpose [13]. ICH Q14 complements this by providing guidance on the science- and risk-based development of analytical procedures, including the establishment of system suitability criteria and analytical control strategies [14]. Together, these guidelines provide a recognized standard against which analytical procedures can be evaluated, facilitating reproducibility and comparability across laboratories and institutions. By developing a validated functional productivity assay within this framework, we aimed to provide the CF biomanufacturing community with a test that is not only scientifically rigorous but also structured in a manner that supports broader adoption and cross-laboratory comparability.

In the absence of a validated functional productivity assay, maintaining consistent CFPS performance across extract lots may lead to undesired deviations in manufacturing consistency and product quality. To address this gap, we developed and validated an *in vitro* superfolder green fluorescent protein (sfGFP) productivity assay (*iv*-sfGFPpA) following the principles established in the ICH Q2(R2) and ICH Q14 guidelines for analytical procedure development and validation [13, 14]. sfGFP was selected as the target molecule for assay development because sfGFP is a widely utilized reporter to assess productivity in CFPS systems due to the protein's rapid folding kinetics and stability in crude cell lysates [15, 16]. However, despite the widespread use of sfGFP as a productivity reporter, to our knowledge, no established standard methodology or reference material exists for the preparation of sfGFP for use as an analytical standard in CFPS systems. In practice, sfGFP is typically produced in-house by individual laboratories using variable materials and methods, introducing an additional and often overlooked source of inter-laboratory variability. For example, differences in downstream purification protocols can yield in-house reference standards with varying levels of monomeric purity, while disparate formulation buffers can introduce chemical artifacts and alter background fluorometric measurements. Consequently, these discrepancies compromise the accuracy of the measurements, obscuring true differences in extract productivity. To differentiate our approach from conventional laboratory preparations, we manufactured an in-house sfGFP reference material governed by a control strategy. By monitoring sample suitability against predefined acceptance criteria prior to use, we ensure consistent material quality to support the development of a validated analytical framework.

Important material attributes, as conceptually defined in ICH Q8(R2) [17], of *E. coli* cell extracts are key targets for monitoring in CF manufacturing workflows [18]. This fluorescence-based analytical framework provides a quantitative productivity measurement to establish and monitor these attributes. Furthermore, to support batch-to-batch comparability and standardization efforts across the biomanufacturing community, all methodological protocols and validation data from this study have been made free and openly available. Ultimately, this analytical tool is intended to support stakeholder adoption of CF technologies by demonstrating a measurement framework to advance reliable extract-based biomanufacturing.

For this work, the *iv*-sfGFPpA fluorescence-based, CF reaction assay was designed to provide a quantitative readout of *E. coli* extract translational yield for CF therapeutic protein manufacturing purposes. In brief, the assay works by combining a cell extract with a consistent reaction master mix and a plasmid DNA template encoding sfGFP. Upon incubation, the extract's endogenous transcription and translation machinery drives sfGFP synthesis, and the measured fluorescence signal (excitation 480 nm / emission 520 nm) serves as a direct functional readout of extract productivity. To support quantitative accuracy and cross-measurement comparability, the purified in-house sfGFP reference material was produced and characterized, enabling construction of a sfGFP concentration curve against which extract-derived sfGFP fluorescence was interpolated. This in-house sfGFP reference material also underpins the system suitability tests used to qualify instrument and assay performance prior to each analytical measurement. Together, these components form an integrated analytical framework in which the fluorescence readout reflects the protein-synthesis capacity of a given extract batch, enabling lot-to-lot comparisons and identification of underperforming extract prior to its use in CF manufacturing workflows.

Results

2.1 Manufacture and characterization of the in-house sfGFP reference material

To support analytical accuracy and reproducibility of the *iv*-sfGFPpA, a recombinant his-tagged sfGFP was manufactured in-house using a traditional cell-based expression and purification process, then characterized for use as a reference material for this study. This recombinant sfGFP was expressed in *E. coli* using a 1 L-scale culture (upstream process) and a three-step chromatographic workflow (downstream process) (Fig. 1a). This manufacturing process yielded sfGFP formulated in a storage buffer (100 mmol/L NaCl, 25 mmol/L Tris-HCl, pH 6.5) with an overall downstream recovery of 71%.

Identity, purity, and the physicochemical properties of sfGFP were evaluated for three independent 4 L batches to qualify the in-house reference material against sample suitability criterion as defined in the FDA Guidance to Industry Q14 titled, “Analytical Procedure Development” [14]. Coomassie-stained SDS–PAGE revealed a single band at the expected molecular weight ((~ 27 to 29) kDa) under both reducing and non-reducing conditions (Fig. 1b). In-gel fluorescence imaging of the sfGFP band confirmed

emission at 520 nm following 488 nm excitation, with no signal detected at 620 nm following 561 nm excitation (Fig. 1c). Impurities and aggregates were assessed by non-reducing capillary electrophoresis–SDS (ceSDS) and size-exclusion chromatography (SEC–UPLC). The ceSDS profiles indicated > 97% purity (Fig. 1d), and SEC–UPLC demonstrated > 95% monomeric content (Fig. 1e). These data support consistent manufacture of sfGFP across three independent batches, with > 97% purity by ceSDS and > 95% monomeric content by SEC-UPLC, indicating minimal low- and high-molecular-weight species.

The sample suitability criteria were defined to establish consistent quality of the in-house sfGFP reference material used across all validation experiments. An sfGFP batch was qualified for use if the batch met the following criteria: fluorescence identity (excitation 488 nm/emission 520 nm), a single band at ~ 29 kDa by Coomassie-stained SDS–PAGE, purity > 94% by ceSDS, and a stock protein concentration of (30 to 40) mg/mL as determined by a Bradford protein assay. To generate in-house sfGFP reference material for analytical method development and validation, we manufactured three 4-liter batches of sfGFP, which all met the predefined criteria (Supplementary Table 1).

2.2 System suitability test development

To define baseline assay performance and system suitability criteria, a system suitability test (SST) was developed using the in-house sfGFP reference material diluted in phosphate-buffered saline (PBS). Dilutional series of three separate in-house reference material batches were generated and evaluated by two analysts on different days to determine the sensitivity limits of the fluorescence readout. For the purposes of this study, the term 'run' denotes a discrete, independent analytical execution comprising multiple technical replicates performed under identical conditions, consistent with its usage in FDA guidance documents governing analytical procedure validation [13]. The limit of detection (LOD) was established at ≤ 0.3 mg/L (signal-to-noise ratio [S/N] ≥ 3), and the limit of quantitation (LOQ) was established at 3.0 mg/L (S/N ≥ 10) (Supplementary Table 2).

For routine quantification, a restricted six-point concentration curve ((3.0 to 600) mg/L) was selected to define the linear range used for SST evaluation. Linear regression of this curve yielded R^2 ≥0.98 across runs (observed range: 0.987 to 0.996) (Supplementary Table 2). Intermediate precision across this range was characterized by relative standard deviation (RSD) < 25% at each concentration level (Supplementary Table 3).

The stability of the sfGFP diluted in the SST matrix was evaluated under freeze–thaw stress. The S/N ratios for the two lowest concentrations ((3.0 and 6.0) mg/L) decreased by 50% after a single freeze–thaw cycle at − 80°C (Supplementary Fig. 2). Based on these observations, the in-house reference material was freshly diluted during the execution of each validation assay.

The SST acceptance criteria were defined prior to analysis of CF extracts (Table 1). An assay plate was considered acceptable for use in this study when the instrument met the manufacturer's fluorescence and performance specifications, the six-point protein concentration curve achieved R^2 ≥0.98, the 3.0 mg/L concentration maintained a S/N ≥ 10, and the technical replicate variability remained < 15% RSD. In

addition, minimum and maximum fluorescence intensity (relative fluorescence units; RFU) bounds were established for each protein concentration level to monitor day-to-day instrument performance.

Table 1
iv-sfGFP SST criteria and acceptance criteria

Description	Acceptance criteria	Batch IDs: B05012025, B06272025, and B07132025
Manufacturer's FL1 plate validation	Meets manufacturer's specifications	Pass
Fluorometric measurements (Ex 480 nm/Em 520 nm) of a 6-point sfGFP standard series (600, 300, 60, 30, 6, and 3 mg/L) prepared in PBS.	Linearity: $R^2 \geq 0.98$ for the 6-point range (600, 300, 60, 30, 6, and 3 mg/L)	Pass
	LOQ (3 mg/L): S/N ≥ 10	Pass
	Precision: % RSD per concentration < 15%	Pass
	Fluorescence intensity bounds (mean ± 2 SD) at each protein level: 600 mg/L: 6.32E + 07 to 1.04E + 08 300 mg/L: 3.55E + 07 to 6.54E + 07 60 mg/L: 7.20E + 06 to 1.49E + 07 30 mg/L: 2.80E + 06 to 7.58E + 06 6 mg/L: 4.10E + 05 to 1.18E + 06 3 mg/L: 2.10E + 05 to 5.4E + 05	Pass

2.3 Preliminary qualification of cell-free reaction components

Preliminary sample suitability criteria were established to qualify all components to reduce the likelihood that observed variability reflected degradation or inconsistency of key biochemical inputs rather than analytical performance. The CFPS reaction matrix is complex, comprising 36 commercially sourced small molecules combined with biologically derived *E. coli* extract and a plasmid DNA template. Establishing a state of control over these inputs was a necessary quality prerequisite for our study. For

the commercially sourced biochemical reagents, baseline quality was verified by deferring to the manufacturers' Certificates of Analysis (COA). However, commercial specifications may not yet fully capture the attributes that impact CF manufacturing, which warrants further investigation. For this report, internal specifications were developed to characterize these in-house manufactured biological components.

E. coli cell extracts were evaluated against two primary suitability criteria prior to use (Supplementary Table 4). The first criterion was that the total protein concentration was (measured via a commercial Bradford assay) within an operating acceptance range of (28 to 38) mg/mL. The assay concentration range was suitable for the extracts to support molecular crowding compatible with translational productivity, while limiting greater viscosities that can hinder microscale mixing [18–22]. Second, extract batches were assessed for baseline functional activity by confirming synthesis of detectable sfGFP after a 24 h incubation at 25°C with an appropriate DNA template and reaction master mix (Supplementary Fig. 3a).

Plasmid DNA templates were characterized and reported as previously described to support nucleic acid purity and structural integrity [23]. Concentration and purity were estimated by UV spectroscopy, with a target A260/A280 ratios of approximately 1.8 to 1.9 that would be consistent with high DNA purity (Supplementary Table 5). In addition, microfluidic electrophoresis was used to assess structural integrity. For use during this validation study, the DNA template was expected to present a single, well-defined peak at the anticipated size (~ 3520 bp) comprising > 95% of the total area under the curve (Supplementary Table 5 and Supplementary Fig. 3b). Collectively, these preassessments were designed to ensure that any variability observed during development of the validated method could be attributed to analytical performance rather than to the quality or stability of the biochemical inputs used in the assay.

2.4 Development of the validated analytical method

Following selection of SST acceptance criteria, analytical procedure development was performed to evaluate the performance of the *iv-sfGFPpA* for quantifying sfGFP in CF reaction matrix containing *E. coli*-derived extracts. To align the procedure development with validation following ICH Q2(R2) recommendations, a spike-recovery study was designed to assess linearity, limits of detection and quantitation, accuracy, repeatability, intermediate precision, specificity, and robustness of the *iv*-sfGFPpA.

Linearity, sensitivity, repeatability, and intermediate precision were assessed using a 12-point sfGFP spike series prepared in CF reaction matrix containing a control DNA plasmid. The series spanned (0 to 200) mg/L of sfGFP ((0, 0.20, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200) mg/L). Data were collected across 27 independent analytical runs performed by two analysts and incorporated three *E. coli*-derived extract batches and three in-house sfGFP reference material batches. Each concentration level was analyzed in technical octuplicate within each run for intra-assay precision (Supplementary Table 6). The technical replicates for each of the sfGFP concentrations were within the predefined acceptance criteria of < 10% RSD. The highest observed RSD was 6.9%.

2.4.1 Parameters: precision, limits of detection and quantitation, and linearity with range

To define the assay's sensitivity limits within the complex biological CFPS reaction matrix, S/N ratios were evaluated across lower protein concentrations from the 27 independent validation runs (Supplementary Table 7). LOD was empirically established at 0.39 mg/L, which yielded a mean S/N ratio of 3.71 and met the predefined criterion (S/N ≥ 3.0). The LOQ was established at 3.125 mg/L, yielding a mean S/N ratio of 25.68 and meeting the predefined criterion (S/N ≥ 10.0).

Following establishment of the LOQ, linearity was assessed using a 6-point working range spanning (6.25 to 200) mg/L of sfGFP ((6.25, 12.5, 25, 50, 100, and 200) mg/L) (Supplementary Table 8). The starting concentration was selected to be 6.25 mg/L, because the 3.125 mg/L concentration did not meet the predefined intermediate precision RSD of < 25% (Supplementary Table 7). The regression across this series met the predefined acceptance criterion (R^2 ≥0.98), with a mean coefficient of determination of 0.998 (SD = 0.001) across all validation runs.

2.4.2 Parameter: intermediate precision

To evaluate intermediate precision, the variability in fluorescence measurements was analyzed throughout a fully crossed experimental design incorporating two analysts, three cell extract batches, and three sfGFP reference material batches. The predefined acceptance criterion specified that relative standard deviation (RSD) must remain ≤ 25% at each protein concentration level within the working range of sfGFP ((6.25 to 200) mg/L). This criterion was applied both to individual extract batches and to the aggregated dataset. As detailed in Supplementary Table 9, the assay met the predefined intermediate-precision criterion across the working range. For the aggregated dataset, intermediate precision ranged from 22.92% RSD at 6.25 mg/L to 9.01% RSD at 200 mg/L.

To establish the upper and lower acceptance limits for the analytical procedure, a "mean ± 2 standard deviations formula" was applied to the dataset (Supplementary Table 10). This existing statistical approach as defined in USP < 1010 > Analytical Data Interpretation and Treatment for defining control limits can support quality control and process validation within regulated pharmaceutical industries [13, 24, 25]. Accordingly, the acceptance limits were calculated by applying the mean ± 2 standard deviations formula to the mean fluorometric measurements of the sfGFP and the standard deviations observed at each protein concentration level.

2.4.3 Parameters: repeatability and accuracy

To evaluate repeatability and accuracy, the dataset from the 27 independent analytical runs was analyzed. Each run included technical octuplicates per concentration level, yielding 216 measurements (27 runs × 8 replicates) per protein level. Repeatability was assessed by calculating the within-run RSD, with a predefined acceptance criterion of < 15%. Accuracy was assessed as percent recovery of

measured sfGFP concentration relative to the theoretical spiked value, with a predefined acceptance range of (80 to 120) %.

As indicated in Supplementary Table 11, the assay met the predefined acceptance criteria across the evaluated working range. At 6.25 mg/L of sfGFP, the assay yielded a mean accuracy of 83.0% and a mean repeatability RSD of 10.3%. Across (12.5 to 200) mg/L, accuracy recoveries ranged from 96.0% to 104.9%, with mean repeatability RSDs ranging from 3.2% to 8.4%.

2.4.4 Parameter: specificity

Specificity was evaluated to assess the analytical procedure's capability to quantify sfGFP in the presence of the CF reaction matrix, including non-target proteins and concentrated *E. coli* cell extracts. Fluorescence was monitored using 2 nm increments from the target excitation at 480 nm and target emission at 520 nm to align with the sfGFP spectral profile and to maximize S/N. These settings were selected to separate excitation light from emitted fluorescence and to limit optical contributions from the protein-rich CF reaction matrix, which has background fluorescence at the same excitation and emission wavelengths of the sfGFP reference material.

To assess potential interference, the CF reaction matrix was spiked with sfGFP at the LOQ (3.125 mg/L) and at the upper limit of the working range (200 mg/L). Signals were compared against potential interferents, including a commercial monoclonal antibody (150 kDa), a representative therapeutic protein (~ 30 kDa), and a CF reaction matrix containing a 2× concentration of cell extract. As detailed in Supplementary Table 12, full-spectrum excitation and emission scanning indicated minimal fluorescence for the background matrix and non-target proteins at the target wavelengths. The presence of these components did not interfere with sfGFP quantification; percent differences between control samples and samples containing interferents remained within the predefined acceptance criterion (≤ 20%) at both concentration levels.

2.4.5 Parameters: robustness

Robustness was evaluated by assessing the effect of small, deliberate variations in reasonable laboratory handling conditions. A bracketing approach was used to evaluate thermal stress and ambient light exposure at two sfGFP concentrations: 3.125 mg/L (LOQ) and 200 mg/L (upper limit of the working range). Samples were incubated at 25°C and 37°C for up to 6 h while exposed to ambient laboratory light to simulate extended handling.

As described in Supplementary Table 13, fluorescence intensity remained stable under the handling conditions evaluated. The combined RSD for fluorescence intensity (RFU) and S/N across all time points at 25°C and 37°C remained below the predefined 25% criterion, with a maximum combined variance of 20.09% at the LOQ. Exposure to 80°C at the indicated timeframes resulted in substantially increased signal variability (RSD > 70%), consistent with loss of sfGFP fluorescence under high thermal stress.

Freeze–thaw stability was evaluated by subjecting the working concentration series ((6.25 to 200) mg/L) to four consecutive freeze–thaw cycles (− 80°C to ambient temperature). As shown in Supplementary Table 14, RFU remained consistent across cycles. The maximum observed RSD across cycles was 9.59% for RFU and 16.05% for S/N, which met the predefined < 25% acceptance criteria.

2.4.6 Proposed performance specifications for routine monitoring

Based on the performance of the analytical procedure (Supplementary Tables 6 to 14), a validation dataset was summarized to define the proposed performance specifications for routine use of the *iv*-sfGFPpA (Table 2). Based on the observed accuracy and precision, the method demonstrated a reportable range of 6.25 to 200 mg/L. For routine testing, the matrix-based 6-point reference curve was evaluated against the system suitability criteria described above, including a coefficient of determination (R^2) ≥ 0.98. Sensitivity was monitored by confirming that the lowest concentration level (6.25 mg/L) maintained a S/N ratio ≥ 10. Repeatability was monitored by assessing the RSD across technical replicates within a run (< 15%), and intermediate precision across analysts and days (≤ 25%). In addition, fluorescence intensity bounds (mean ± 2 SD) were defined for each concentration-curve level to support monitoring of day-to-day instrument and matrix performance. Sample handling conditions evaluated during robustness testing (ambient laboratory light exposure; temperatures up to 37°C for up to 6 h; and up to four freeze–thaw cycles from − 80°C) were used to inform recommended limits for routine handling of analytical samples. Together, these criteria support consistent application of the assay for monitoring extract productivity in the workflows evaluated here.

Table 2
Analytical Method Validation Performance Summary for the *iv*-sfGFPpA.

Validation Parameter	**Predefined Acceptance Criteria**	**Observed Performance**	**Status**
Intra-assay Precision (Repeatability)	RSD < 10% per run	Range: 0.40% to 6.9% RSD	Pass
Limit of Detection	S/N Ratio ≥ 3.0	0.39 mg/L (mean S/N = 3.71)	Pass
Limit of Quantitation	S/N Ratio ≥ 10.0	3.125 mg/L (mean S/N = 25.68)	Pass
Linearity (Working Range)	R^2 >0.98 (6.25 to 200 mg/L)	mean R^2 = 0.998	Pass
Intermediate Precision	RSD < 25% (6.25 to 200 mg/L)	Maximum RSD = 22.92	Pass
Repeatability	RSD < 15% (6.25 to 200 mg/L)	RSD Maximum = 10.3%	Pass
Accuracy	80 to 120% (6.25 to 200 mg/L)	Maximum = 104.9% Minimum = 83.0%	Pass
Specificity	≤ 20% interference from matrix/non-target proteins	No significant interference detected (Against 150 kDa, 30 kDa, and 2x Extract conditions)	Pass
Robustness (Thermal/Light)	RSD of RFU and S/N < 25% after 6 h at 25°C and 37°C	Maximum combined RSD = 20.09% (Passes criteria at handling temps)	Pass
Robustness (Freeze-Thaw)	RSD of RFU and S/N < 25% after 4 cycles (-80°C to Ambient)	Maximum RFU RSD = 9.59%, Maximum S/N RSD = 16.05%	Pass

The specifications for the *iv*-sfGFPpA assay were derived from the developmental validation dataset to ensure the method remained fit-for-purpose for the routine quantification of *E. coli* extract productivity as shown in Table 3. The analytical procedure was validated for a reportable dynamic range of (6.25 to 200) mg/L. To deem an analytical run valid, the linear regression of the sfGFP concentration curve must demonstrate a coefficient of determination (R^2) of ≥ 0.98. The LOQ was specified as having the lowest concentration exhibiting a signal-to-noise ratio of ≥ 10, while the LOD corresponded to a ratio of ≥ 3. For assay precision and repeatability, the RSD for calculated concentrations within the linear range must be ≤ 25%, ensuring consistent performance across different analysts and instrument runs. The method specifications also encompassed sample handling constraints; the assay was valid for validation

samples (sfGFP diluted in CF reaction matrix) subjected to a maximum of four freeze-thaw cycles (-80°C) and withstood ambient laboratory conditions (light exposure and temperatures up to 37°C) for a duration not exceeding 6 h. These combined criteria constituted the final quality specifications for the deployment of the *iv*-sfGFPpA in a quality control setting.

Table 3
Validation Specifications and Acceptance Criteria for the *iv*-sfGFPpA.

Description	Acceptance criteria	Results
System Suitability Tests	Meets system suitability test criteria	Pass/Fail
6-point reference material gradient diluted in matrix. (6.25, 12.5, 25, 50, 100, and 200 mg/L)	The linearity correlation coefficient ≥ 0.98 using a 6-point range (200, 100, 50, 25, 12.5, and 6.25 mg/L)	Pass/Fail
	Lowest concentration (6.25 mg/L) has a S/N ratio ≥ 10.	Pass/Fail
	The RSD across triplicate samples within the protein levels should be < 15%. (200, 100, 50, 25, 12.5, and 6.25 mg/L)	Pass/Fail
	The mean sfGFP fluorescent intensity (mean ± 2 S.D.) values at each protein level are as follows. 200 mg/L range = 2.64E + 07 to 3.72E + 07 100 mg/L range = 1.40E + 07 to 2.23E + 07 50 mg/L range = 6.92E + 06 to 1.02E + 07 25 mg/L range = 3.21E + 06 to 5.19E + 06 12.5 mg/L range = 1.37E + 06 to 2.63E + 06 6.25 mg/L range = 4.79E + 05 to 1.34E + 06	Pass/Fail

2.5 Partial optimization of CFPS reaction conditions

Although the fluorometric readout of the *iv*-sfGFPpA was validated using the CF reaction matrix spiked with in-house sfGFP reference material following ICH guidance, the routine use of the assay to monitor extract performance also benefits from minimizing intrinsic variability in the underlying CFPS reaction. To identify operating conditions with improved reproducibility, microscale reaction volumes ((50, 100,

and 200) µL) and incubation temperatures ((25, 37, and 50) °C) were evaluated to assess changes in intermediate precision of the assay. Three analysts performed three independent runs with triplicate technical replicates using single lots of *E. coli* extract, plasmid DNA, and master mix reagents to isolate effects attributable to environmental and volumetric factors over a 24-h incubation.

As summarized in Table 4, incubation temperature affected both sfGFP productivity and inter-analyst variability. CF reactions incubated at 50°C produced minimal fluorescence, consistent with reduced activity of the *E. coli* transcriptional and translational machinery under elevated thermal conditions. Incubation at 37°C supported sfGFP synthesis but was associated with higher inter-analyst variability across tested volumes (RSD range: 51.73 to 63.67%), indicating increased sensitivity to handling differences and/or more rapid reagent depletion at this temperature.

Incubation at 25°C provided higher fluorescence signals with lower variability (RSD range: 12.50 to 19.86%) under the conditions evaluated. Among the tested configurations, the 50 µL reaction volume at 25°C yielded the lowest RSD (12.50%) while maintaining high fluorescence signals. Consequently, subsequent *iv*-sfGFPpA experiments in this study were performed using 50 µL reactions incubated at 25°C to support improved reproducibility and to reduce the likelihood that assay-to-assay variability reflected reaction-condition effects rather than extract-to-extract differences.

Table 4
Evaluation of CFPS Reaction Conditions

Time (h)	Temperature	Volume (µL)	Mean RFU (n = 9)	S.D.	RSD (%)
0	N/A		1.09E + 04	1.44E + 03	13.23
24	25	50	1.14E + 06	1.43E + 05	12.50
		100	1.08E + 06	1.67E + 05	15.49
		200	1.13E + 06	2.24E + 05	19.86
	37	50	6.54E + 05	4.16E + 05	63.67
		100	5.21E + 05	3.26E + 05	62.61
		200	4.19E + 05	2.17E + 05	51.73
	50	50	4.21E + 04	3.11E + 03	7.39
		100	4.36E + 04	3.89E + 03	8.93
		200	4.46E + 04	5.63E + 03	12.63

2.6 Extract performance monitoring and scale-up analysis

To demonstrate proof-of-concept for the use of the *iv*-sfGFPpA for monitoring the functional performance of *E. coli* cell extracts, batches of extract manufactured at two scales were evaluated: a 1 L laboratory scale at the FDA and a 50 L pilot scale at the U.S. Army Combat Capabilities Development

Command Chemical Biological Center (DEVCOM-CBC). This inter-laboratory comparison assessed the assay's ability to quantify productivity differences between extract lots during process scale-up.

To establish a laboratory-scale baseline, three independent 1 L batches were analyzed. As summarized in Table 5, the assay differentiated between extracts with measurable sfGFP productivity and an extract whose sfGFP synthesis signal was below the LOQ. Batch B0520E0521F_2025 yielded 86.65 mg/L (1.13% RSD), followed by batch B0716E0830F_2025 at 67.14 mg/L (1.85% RSD). In contrast, batch B0529E0708F_2025 produced an sfGFP synthesis signal below the LOQ under the assay conditions, indicating that the quantity of sfGFP synthesized by this extract was insufficient for quantification within the validated working range.

To further assess inter-site use, three 50 L pilot-scale batches (CBC-P1, -P2, and -P3) produced by DEVCOM-CBC were evaluated. As described in Table 5, one pilot batch (CBC-P1) yielded 84.53 mg/L (3.40% RSD) sfGFP, which was within the range observed for the laboratory-scale batches in this dataset. The remaining two pilot batches (CBC-P2, and -P3) produced an sfGFP synthesis signal below the LOQ using the established assay conditions.

Together, these data indicate that the *iv*-sfGFPpA can quantify extract productivity across batches and can distinguish lots with fluorescence below the quantitation limit under the assay conditions. Such measurements may support comparative assessment of extract performance during laboratory-to-pilot scale-up efforts.

Table 5
Productivity Assessment of Laboratory and Pilot-Scale *E. coli* Cell Extracts.

Manufacturing scale	Extract batch ID	Replicates	Mean sfGFP (mg/L)	SD	% RSD	Assay outcome
Laboratory scale	B0520E0521F	8	86.65	0.98	1.13%	Quantified
1 L	B0716E0830F	8	67.14	1.24	1.85%	Quantified
	B0529E0708F	8	<LOQ	N/A	N/A	Below LOQ
Pilot scale	CBC-P1	8	84.53	2.87	3.40%	Quantified
50 L	CBC-P2	8	<LOQ	N/A	N/A	Below LOQ
	CBC-P3	8	<LOQ			Below LOQ

N/A = not applicable.

Discussion

The use of CFPS for decentralized or on-demand biomanufacturing is influenced by the quality and reproducibility of key raw materials, particularly the cell extract. Although the open nature of CFPS reactions enables direct manipulation of transcription conditions and addition of reagents, the

biochemical complexity of the extract introduces substantial lot-to-lot variability in yields. Fluorescent reporter-protein readouts are commonly used to assess CFPS performance, but these approaches have rarely been evaluated using structured analytical validation studies. Here, we describe the development of an ICH Q2(R2)-informed validation of an *in vitro* sfGFP productivity assay, *iv*-sfGFPpA, intended to quantify extract productivity in an *E. coli*-based CFPS reaction format. These results, and the development of an in-house CF manufacturing process to identify parameters affecting CF manufacturing consistency and product quality, offer a practical resource for CF manufacturing stakeholders.

An important aspect of this work was the application of a structured analytical validation approach to a multicomponent biological matrix. By generating characterized in-house sfGFP reference material and implementing SSTs, we aimed to reduce variability attributable to the fluorescence measurement workflow and to support comparability of extract performance assessments. The validation data indicated that fluorometric quantification of sfGFP was suitably linear, accurate, and precise across a relatively wide evaluated working range ((6.25 to 200) mg/L). In addition, specificity and robustness studies indicated that the selected excitation/emission settings provided a signal that was principally attributable to sfGFP, with limited interference from the protein-dense *E. coli* matrix under the tested conditions. Together, these data support the interpretation that observed differences in productivity across extract lots primarily reflect differences in extract performance rather than systematic assay drift or matrix interference.

In addition to validating the fluorometric readout, this study evaluated CF reaction conditions that may influence assay-to-assay variability. Under the conditions tested, incubation at 37°C was associated with higher inter-analyst variability in microscale reactions, whereas incubation at 25°C reduced variability while maintaining high fluorescence signals. The temperature-induced differences in the reaction may be caused by the reported temperature-induced chaperone overload, slowing translation for proper folding, or the rapid degradation of supplements, such as amino acids and energy sources [26–29]. The reaction format (50 µL at 25°C) used for measurement reduced the likelihood that handling-related effects are misinterpreted as extract-to-extract differences in this study.

The iv-sfGFPpA was intentionally designed as a 24 h endpoint assay, rather than a time-course measurement, as this approach was potentially better-suited to the intended application of routine extract qualification in a quality control setting. A single endpoint readout minimized handling steps and time-sensitive sampling events, thereby reducing potential sources of inter-analyst variability, consistent with the precision improvements observed when reaction conditions were partially optimized in this study. Furthermore, the rapid folding kinetics and stability of sfGFP in crude lysates supported the use of an accumulated endpoint fluorescence signal as a reliable integrative measure of total extract productivity over the reaction period [15, 16]. Complementary approaches that capture transcription and translation dynamics over the course of a reaction, such as nucleic acid-based assays [11], may also provide additional mechanistic insight into the kinetics of extract depletion and could be valuable for

process development applications, where understanding reaction dynamics is important beyond the reported endpoint productivity metric.

While the validated *iv*-sfGFPpA used here provided a robust framework for assessing extract performance, an important limitation was that the validated scope was strictly bound to the specific operating conditions established in this study. The precision, accuracy, and linearity demonstrated herein reflect the performance of E. coli-derived extracts specifically within the defined analytical parameters, including the established total protein concentration range, reaction conditions, and reaction mixtures. Consequently, any significant deviation from these conditions, such as the utilization of alternative microbial host strains, distinct extract preparation methodologies, or modified reaction conditions and temperatures, falls outside the scope of this current analytical procedure development reported in this study. Stakeholders seeking to apply this assay to divergent CF platforms should perform appropriate revalidation or bridging studies to ensure the method remains fit-for-purpose under modified operational conditions. It should also be noted that the energy mix composition and DNA template concentration were held constant across all extract types evaluated in this study. To ensure a controlled comparative investigation, the energy mix composition and DNA template concentration were held constant across all extract types evaluated in this study. However, because these parameters are known to influence CFPS productivity in an extract-dependent manner, they may require independent optimization, when the extract preparation method differs [4, 27].

Two additional limitations also merit consideration when applying the *iv*-sfGFPpA to inform CFPS production decisions. First, this assay was optimized and validated at 25°C, whereas some CFPS production runs are conducted at 37°C. As demonstrated in Section 2.5, incubation at 37°C was associated with substantially higher inter-analyst variability in microscale reactions, which may indicate the current conditions were not fully optimized for CF manufacturing. Consequently, extract productivity measurements generated at 25°C may not directly predict extract performance under production-relevant thermal conditions, and users operating CFPS platforms at 37°C should consider whether bridging data at the production temperature are needed to support result interpretation. Second, sfGFP is a relatively small, rapidly folding protein (~ 27 kDa) that has been widely adopted as a fluorescent reporter protein for CFPS because of its favorable folding kinetics and stability in crude lysates [15, 16]. However, the translational demands placed on the extract by larger or more complex target proteins, including those requiring chaperone assistance, disulfide bond formation, or post-translational modifications, may differ substantially from those captured by the sfGFP readout. As such, the *iv*-sfGFPpA should be interpreted as a measure of general translational capacity rather than a direct predictor of yield for any specific target protein, and its predictive value for larger or structurally complex proteins may warrant further evaluation.

The scale-up dataset revealed that all six extract batches, including two of three laboratory-scale lots and three of three pilot-scale lots, produced sfGFP fluorescence. However, two pilot-scale lots were below the LOQ under the assay conditions evaluated. While this frequency may appear high, it is consistent with the known sensitivity of CFPS extract productivity to upstream process parameters,

including fermentation growth rate, harvest timing, lysis efficiency, and run-off reaction conditions, any of which can vary during early-stage process development and scale-up campaigns [5–7]. In this context, the sub-LOQ result is itself an informative outcome rather than an assay failure: it indicates that the *iv*-sfGFPpA can discriminate between extract lots with measurable translational activity and those whose productivity falls below the assay's detection threshold under the defined reaction conditions. From a manufacturing quality control perspective, a sub-LOQ result could trigger a structured root cause investigation for the material qualification or the upstream manufacturing process to identify the source of reduced extract performance. Importantly, the assay does not distinguish between an extract with genuinely low translational capacity and one that may perform adequately under modified CF reaction conditions. These observations underscore the value of the *iv*-sfGFPpA as an early-stage screening tool that can flag underperforming lots and prioritize process development efforts, while also highlighting the need for a defined disposition protocol that contains criteria for lot rejection, retest, or conditional use to support its integration into a formal quality control workflow.

This inter-laboratory scale-up assessment further illustrates the assay’s usefulness for comparing extract performance across manufacturing scales. In this dataset, the *iv*-sfGFPpA quantified productivity for the laboratory-scale extracts and identified several pilot-scale batches with fluorescence below the quantitation limit. These observations highlight that extract manufacturing outcomes may vary across the development of pilot-scale campaigns and indicate that the assay can provide a quantitative readout that may be used to support investigations of process conditions associated with lower-performing batches, which would be beneficial in both the research and commercial settings.

It is worth noting the intended utility of this work for the broader CF biomanufacturing community. The *iv*-sfGFPpA is not intended to prescribe a universal set of reaction conditions for all CFPS systems. The specific parameters identified represent conditions partially optimized for the *E. coli*-based system evaluated in this study and should be understood as a worked example rather than a universal recommendation. The primary contribution of this work is the analytical framework itself: a structured, ICH Q2(R2)-informed approach to developing, optimizing, and validating a fluorescence-based productivity assay for monitoring extract batch-to-batch variability. CF stakeholders may adopt such a template framework using sfGFP or an alternative fluorescent reporter appropriate for their system and to perform their own reaction condition optimization and analytical validation prior to routine use. The validation parameters, acceptance criteria, and system suitability tests described here provide a practical starting point that may be adapted to other extract types, reaction formats, and production scales. Overall, in this work, we provide an example of a reproducible, data-driven approach to establishing confidence in extract quality before committing material for a manufacturing campaign.

Conclusion

Cell-free protein synthesis applications that rely on decentralized or on-demand production can be influenced by the consistency of important raw materials. In this study, we developed and validated an *in vitro* sfGFP productivity assay following ICH Q2(R2) to guide the establishment of an in-house *E. coli*-

based cell-free manufacturing platform. This analytical procedure demonstrated suitable linearity, accuracy, and intermediate precision across a working range of (6.25 to 200) mg/L. Stress testing further supported method specificity in the presence of matrix components and indicated adequate robustness under the benchtop handling and freeze/thaw conditions evaluated.

By evaluating reaction parameters (25°C at 50 µL), we selected assay conditions that reduced microscale reaction variability in this experimental setting, supporting interpretation of the fluorescence readout as a measure of lot-to-lot extract performance. The assay was then applied in an inter-laboratory comparison of 1 L laboratory-scale batches and a 50 L pilot-scale campaign. Under the assay conditions, the *iv*-sfGFPpA quantified productivity for several lots and identified multiple batches with fluorescence below the quantitation limit.

Overall, the *iv*-sfGFPpA provides a method to quantitatively monitor sfGFP productivity in cell extracts within the CFPS workflow evaluated here. The approach may support extract comparability assessments during pharmaceutical manufacturing process development and scale-up by providing consistent productivity readout across lots, sites, and operators.

Materials and Methods

In support of open science and analytical comparability, step-by-step operating procedures with accompanying materials lists have been made freely and publicly accessible through PrecisionFDA.

9.1 Generation and purification of sfGFP reference material

To generate sfGFP reference material, *E. coli* BL21(DE3) (Thermo Fisher Scientific, Waltham, MA) was transformed with a vector for expressing sfGFP (see Section 9.2, below), and ampicillin-resistant colonies were isolated from Luria–Bertani (Research Products International, Mt. Prospect, IL) /agar plates with 100 µg/mL ampicillin (Sigma-Aldrich, St. Louis, MO). Selected colonies were cultured in LB medium with 100 µg/mL ampicillin for screening of sfGFP by biospectrophotometer (Eppendorf, Enfield, CT) using excitation at 488 nm and emission at 520 nm. Colonies possessing the highest sfGFP/OD_{600} ratio were selected and cultured in 2×YT medium supplemented with ampicillin. Seed cultures were incubated overnight at 37°C with orbital shaking at 250 rpm, normalized by optical density at 600 nm, and used to inoculate production cultures. Cells were harvested by centrifugation at 2,200 × g for 30 min, and pellets were stored at − 80°C.

For extraction, frozen pellets were thawed and resuspended in B-PER bacterial protein extraction reagent (Thermo Fisher Scientific) supplemented with EDTA-free protease inhibitor cocktail (Thermo Fisher Scientific) at 5 mL per gram wet cell pellet. Suspensions were gently agitated for 15 min, and lysates were clarified by centrifugation at 2,200 × g for 20 min followed by filtration through a 0.2 µm polyethersulfone membrane (Corning).

sfGFP was purified from clarified lysate using an ÄKTA pure fast protein liquid chromatography system (Cytiva, Marlborough, MA) with sequential immobilized metal affinity chromatography, anion-exchange chromatography, and cation-exchange chromatography. For affinity capture, a HisTrap column (Cytiva) was equilibrated in 20 mmol/L Tris, 150 mmol/L NaCl, and 25 mmol/L imidazole (pH 7.4), and sfGFP was eluted with 300 mmol/L imidazole. The eluate was concentrated using a 10 kDa molecular-weight-cutoff centrifugal filter (MilliporeSigma, Burlington, MA) prior to ion-exchange steps. Final sfGFP-containing fractions were buffer-exchanged into 25 mmol/L Tris, 100 mmol/L NaCl (pH 6.5) using a 15 mL centrifugal filter (MilliporeSigma) and stored at − 80°C.

9.2 Manufacture of DNA templates and cell-free reaction mixtures

To generate the sfGFP DNA template, a pT7-mRNA-RiboJ-5His-sfGFP plasmid was amplified in *E. coli* DH5α competent cells (Thermo Fisher Scientific). Transformed cells were cultured in LB broth supplemented with ampicillin (100 µg/mL). Production cultures were incubated for 18 h at 37°C with orbital shaking at 250 rpm. Plasmid DNA was purified using a Plasmid Maxi kit (QIAGEN, Hilden, Germany). Plasmid concentration and UV absorbance ratios (A260/A280 and A260/A230) were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Plasmid size and integrity were assessed using an automated electrophoresis system (TapeStation; Agilent Technologies, Santa Clara, CA) prior to downstream use.

CFPS reactions were assembled from five custom master reagent mixtures: a 15× master salt solution; a 15× master mix supplying nucleotides and transcriptional substrates (ATP, GTP, UTP, CTP, folinic acid, and tRNA); a 12× master reaction mix containing cofactors and polyamines (NAD, CoA, oxalic acid, putrescine, spermidine, and HEPES); a 1 M master phosphoenolpyruvate energy solution adjusted to pH 7.2; and a 50 mM master amino acid mixture [26]. The amino acid mixture was prepared by sequential dissolution, including heating to 65°C to solubilize L-tyrosine prior to final volume adjustment and titration to pH 6.7. Chemicals were obtained from commercial suppliers (e.g., Sigma-Aldrich, Roche, MP Biomedicals). Prepared mixtures were filtered through a 0.2 µm polyethersulfone membrane, aliquoted, and stored at − 80°C until use.

9.3 Manufacture of *E. coli*-derived cell extracts

To prepare cell extracts, *E. coli* BL21-Rosetta2(DE3) was cultured in 2×YT medium supplemented with ampicillin (100 µg/mL). Seed cultures were used to inoculate 2.5 L Tunair full-baffle shake flasks (IBI Scientific, Dubuque, IA) containing 1 L medium supplemented with a glucose–phosphate buffer to a starting OD_{600} of 0.1. Cultures were incubated at 37°C with orbital shaking at 250 rpm. At OD_{600} 0.5 to 0.6, T7 RNA polymerase expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; RPI Corporation, Mount Prospect, IL). Cultivation continued to OD_{600} ~3.0, and cells were harvested by centrifugation (5,000 × g for 10 min at 4°C) using an Avanti J-E centrifuge (Beckman Coulter, Brea, CA).

Cell pellets were washed three times by resuspension and centrifugation (2,200 × g for 10 min) in cold S30 buffer (14 mmol/L magnesium acetate, 60 mmol/L potassium acetate, 10 mmol/L Tris-acetate, pH 8.2) freshly supplemented with 2 mmol/L dithiothreitol (DTT; RPI Corporation). Washed pellets were flash-frozen on dry ice and stored at − 80°C. For lysis, thawed pellets were resuspended in cold S30 buffer supplemented with DTT (1 mL per g wet cell mass) and lysed using a microfluidizer (Microfluidizer Model LM10, Microfluidics, a subsidiary of IDEX Material Processing Technologies, Middleborough, MA) for one cycle at 10,000 psi [30, 31].

Immediately after lysis, 6.43 µL of additional 0.5 M DTT was added per milliliter of microfluidized bacterial slurry, and the homogenate was clarified by centrifugation (18,000 × g for 10 min at 4°C). The clarified supernatant was transferred to clean vessels and incubated at 37°C with shaking at 250 rpm for 1 h to perform a run-off reaction [32]. The extract was then further clarified by centrifugation (10,000 × g for 10 min at 4°C). Total protein concentration was measured using the Quick Start Bradford Protein Assay with bovine serum albumin standards (Bio-Rad, Hercules, CA) [22]. The extract was aliquoted, flash-frozen on dry ice, and stored at − 80°C until use.

9.4 Cell-free reaction assembly and optimization

To establish assay conditions and assess intermediate precision of the CF reaction, a master reaction mixture was assembled on ice. Cell-free protein synthesis reactions were prepared by combining *E. coli*-derived extract with five custom reagent mixtures: a 15× salt solution, a 15× master mix, a 12× reaction mix, a 1 M phosphoenolpyruvate energy solution, and a 50 mM amino acid mixture. Reactions were initiated by addition of the sfGFP DNA template to a final concentration of 5.81 nmol/L (13.3 ng/µL).

To evaluate reaction volume and incubation temperature for routine extract qualification, the reaction mixture was dispensed at (50, 100, or 200) µL and incubated at (25, 37, or 50) °C, with 4°C included as a negative control. All reactions were incubated with orbital shaking at 750 rpm for 24 h. For fluorescence analysis, samples collected at (0 and 24) h were diluted 1:10 in phosphate-buffered saline (pH 7.2) and transferred to black 96-well microplates (Greiner Bio-One) for immediate measurement. Based on precision and productivity, subsequent validation testing was performed using 50 µL reactions incubated at 25°C.

9.5 Instrument qualification and system suitability test development

Operational qualification of the SpectraMax iD3 multimode microplate reader (Molecular Devices, San Jose, CA) was performed prior to key data acquisitions using a certified SpectraTest FL1 Validation Plate (96-well format) to verify fluorescence performance according to the manufacturer's procedures. Automated plate-reading methods and validation analyses were executed using SoftMax Pro software (version 7.3.1).

To establish SST parameters and evaluate assay linearity, a 12-point serial dilution of sfGFP reference material was prepared in formulation buffer (100 mmol/L NaCl, 25 mmol/L Tris–HCl, pH 6.5). The

concentration curve spanned 600 mg/L to 0.006 mg/L and included a zero-protein blank. Samples (100 µL per well) were plated in octuplicate in black 96-well microplates with clear bottoms (Greiner Bio-One, Monroe, NC). Fluorescence intensity was quantified with excitation at 488 nm and emission at 520 nm using a well-scan read mode (4 points per well).

The 12-point series was used to determine assay sensitivity. The S/N was calculated as F_{sample}/F_{blank}, where F_{sample} is the fluorescence intensity of the sfGFP sample and F_{blank} is the mean fluorescence intensity of the zero-protein blank. The LOD was defined as the lowest sfGFP concentration with S/N > 3.0, and the LOQ as the lowest concentration with S/N > 10 [13]. Linearity was assessed by linear regression of a 7-point subset of the dilution series ((0.6 to 600) mg/L) using GraphPad Prism software. For routine testing in this study, the system suitability criterion used was that the concentration-curve regression achieves $R^2 \geq 0.98$.

9.6 Analytical method validation

The *iv*-sfGFPpA assay was validated following ICH Q2(R2) and ICH Q14 guidelines. To account for the complex biological background of the manufacturing process, validation samples were prepared by spiking sfGFP reference material into CF reaction matrix containing an empty DNA plasmid vector (pLV-IRES-Puro).

Linearity, accuracy, and intermediate precision were assessed using a 12-point sfGFP concentration series ((0 to 200) mg/L) prepared in the reaction matrix. Measurements were collected across 27 independent runs performed by two analysts. Runs incorporated three independent batches of *E. coli*-derived extract and three independent batches of sfGFP reference materials, with each sample analyzed in technical octuplicate. The LOD and LOQ were defined as the lowest sfGFP concentrations yielding S/N > 3 and S/N > 10, respectively, where S/N was calculated as F_{sample}/F_{blank} and F_{blank} is the mean fluorescence intensity of the zero-protein blank.

Specificity was evaluated by full-spectrum fluorescence scanning to assess potential optical interference from the CF reaction matrix and non-target proteins. Reaction matrix was prepared with sfGFP ((3.125 and 200) mg/L) and compared with background controls, including neat cell extract, a 150 kDa monoclonal antibody (200 mg/L), and a ~ 30 kDa therapeutic protein (200 mg/L). Fluorescence spectra were acquired using emission scans from (510 to 550) nm (excitation 480 nm) and excitation scans from (460 to 500) nm (emission 520 nm).

Robustness was assessed by subjecting sfGFP-spiked reaction matrix to thermal and freeze–thaw stress. For thermal stress, samples were incubated at (25, 37, or 80) °C and fluorescence was quantified after (0, 2, 4, and 6) h. Freeze–thaw stability was evaluated across four cycles of freezing at − 80°C and thawing at 25°C.

9.7 Equipment and settings

Gel photo-images in Fig. 1 and Supplementary Fig. 1 were acquired and analyzed using Azure 600 imaging system and software (Azure Biosystems, Dublin, CA). Coomassie-stained gel images were obtained using Coomassie Blue Auto Image setting with Epi-White illumination, while in-gel sfGFP fluorescence was captured using Fluorescence Auto Image Rapid Capture exposure setting detecting emission at 520 nm wavelength following excitation at 488 nm wavelength (green) and emission at 620 nm following 561 nm excitation (red). Fluorescence and visible-light images of *E. coli* extracts (Supplementary Fig. 3a) were acquired using Azure 600 imaging system detecting fluorescence emission at 520 nm after excitation at 488 nm, or visible light (True Color setting). ceSDS analysis was performed using ProteinSimple Maurice OBM instrument (R&D Systems by Bio-Techne, Minneapolis, MN). SEC-UPLC was performed using Waters Acquity H-Class UPLC (Milford, MA) using fluorescence and UV detectors, with UPLC Protein BEH SEC 125A 4.6 × 150 mm 1.7 µm column.

Declarations

Conflict of Interest

The authors have no competing interests to declare or conflicts of interest. The funding source had no part in the study design, data collection/analysis, decision to publish, or preparation of the manuscript. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the United States Food and Drug Administration and the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the United States Government. Certain commercial entities, equipment, or materials may be identified in this document to describe an experimental procedure or concept adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology and Food and Drug Administration, nor is it intended to imply that the entities, materials, or equipment are necessarily the best available for the purpose.

Data availability

All the relevant data are within this publication and the supplementary material.

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Figures

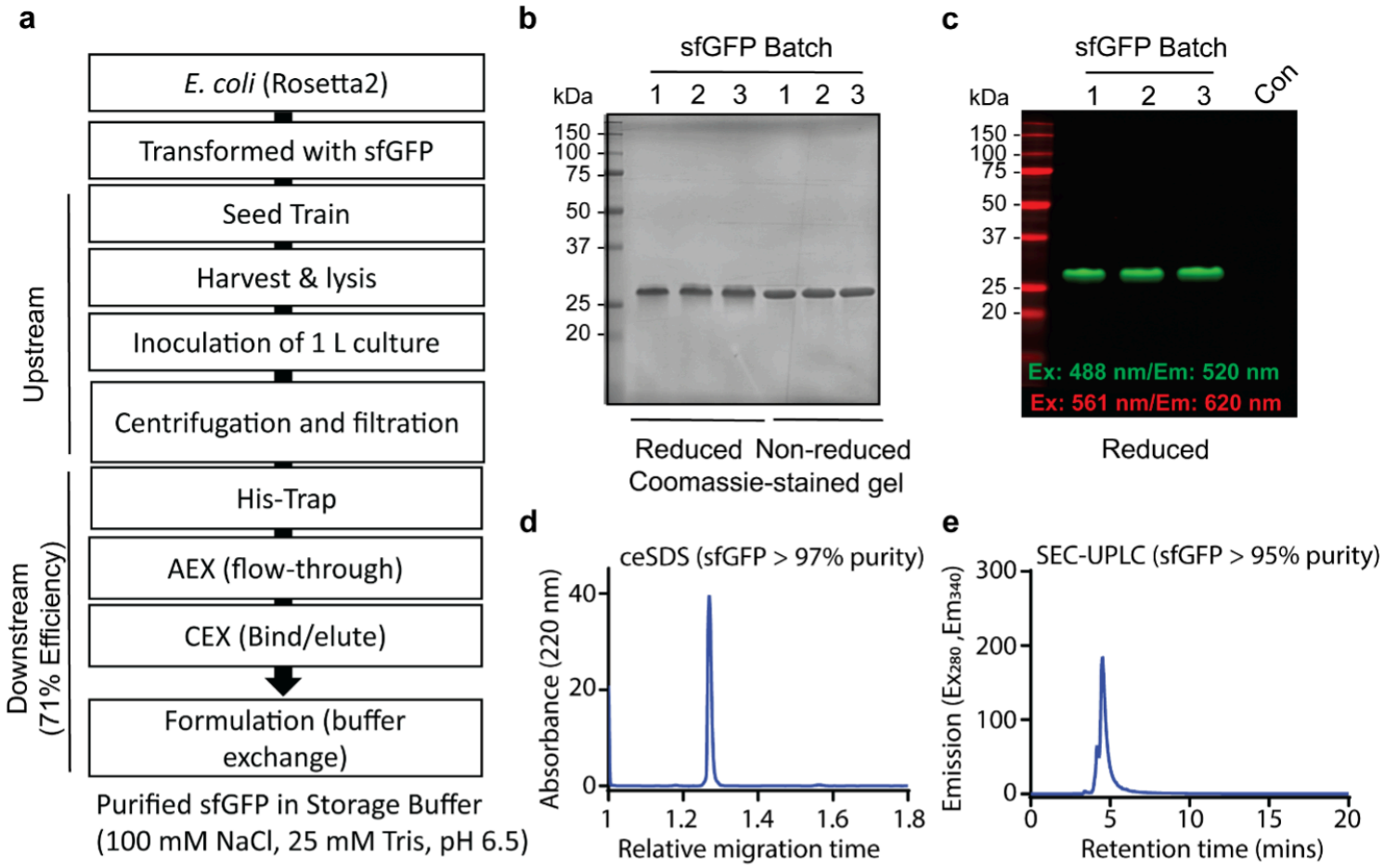


Figure 1

Traditional cell-based biomanufacturing and analytical characterization of recombinant sfGFP reference material. (a) Process flow diagram showing upstream production (1 L scale) and downstream purification using immobilized metal affinity chromatography (HisTrap), anion-exchange chromatography (flow-through), and cation-exchange chromatography (bind-and-elute). The overall downstream recovery was 71%, and purified sfGFP was formulated in 100 mmol/L NaCl, 25 mmol/L Tris (pH 6.5). (b) Coomassie-stained SDS–PAGE of three sfGFP batches (lanes 1–3) under reducing and non-reducing

conditions. (c) In-gel fluorescence imaging of the same three sfGFP batches and a negative control protein on a separate gel from 1(b) showing sfGFP emission at 520 nm (green channel) following 488 nm excitation and no detectable signal at 620 nm (red channel); Control: non-fluorescent irrelevant monoclonal antibody run in the next lane to the right of the sfGFP batches. (d) ceSDS electropherogram showing >97 % purity. (e) SEC–UPLC chromatogram showing >95 % monomeric content and low levels of high-molecular-weight species. Panels (b) and (c) derive from separate gels. The images in the main figure were cropped from the original full-gel captures to focus on the molecular-weight region of interest; no lanes were spliced, rearranged, or selectively grouped, and no post-acquisition contrast adjustment was applied beyond the instrument's automatic image capture. The uncropped, full-length gel images are provided in Supplementary Figure 1.

Supplementary Files

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