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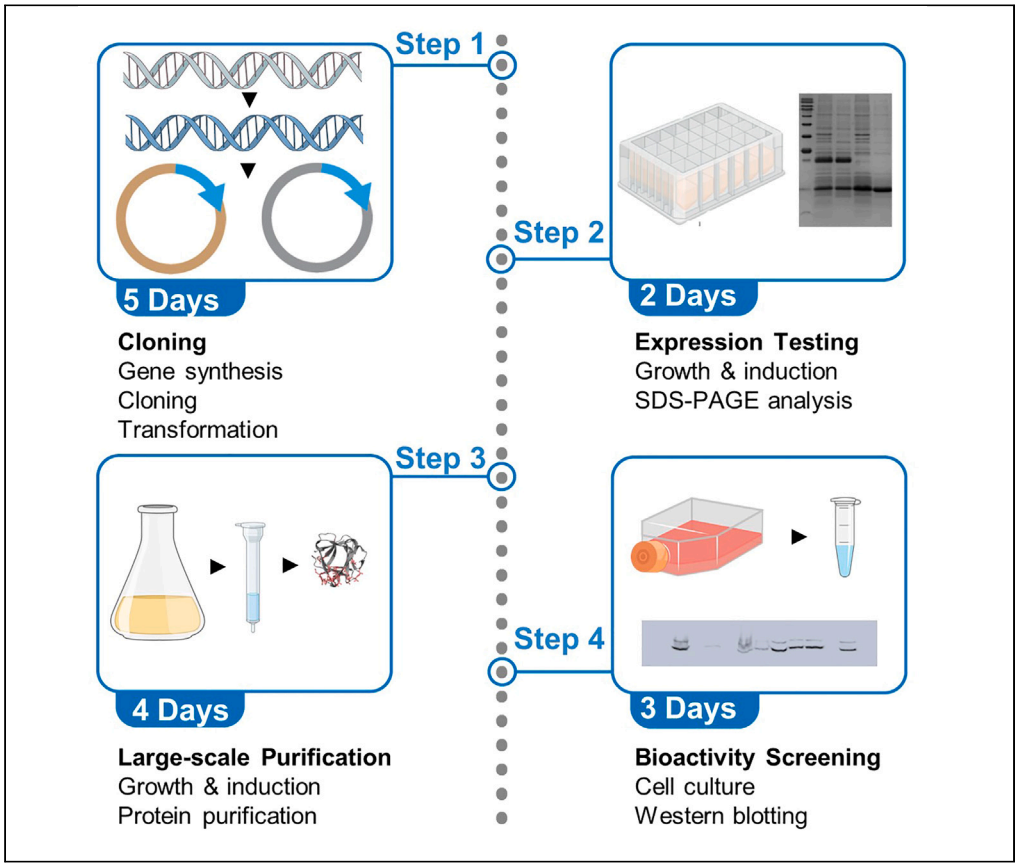
Protein expression and purification of bioactive growth factors for use in cell culture and cellular agriculture



Mitogenic growth factors are major cost drivers in serum-free media, contributing up to 95% of the total cost. Here, we present a streamlined workflow detailing cloning, expression testing, protein purification, and bioactivity screening that allows for low-cost production of bioactive growth factors including basic fibroblast growth factor and transforming growth factor β1. This generalized procedure can be used for multiple families of growth factors with minor modification, and the outputs are bioactive and suitable for cell culture applications.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Standardized protocol for soluble growth factor expression in *E. coli*

Protocol can be readily applied to growth factor orthologs from diverse species

Low production cost is ideal for emerging applications such as cellular agriculture

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Protein expression and purification of bioactive growth factors for use in cell culture and cellular agriculture

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SUMMARY

Mitogenic growth factors are major cost drivers in serum-free media, contributing up to 95% of the total cost. Here, we present a streamlined workflow detailing cloning, expression testing, protein purification, and bioactivity screening that allows for low-cost production of bioactive growth factors including basic fibroblast growth factor and transforming growth factor β1. This generalized procedure can be used for multiple families of growth factors with minor modification, and the outputs are bioactive and suitable for cell culture applications. For complete details on the use and execution of this protocol, please refer to Venkatesan, et al.[1]

BEFORE YOU BEGIN

Mitogenic growth factors are signaling proteins that bind to receptors localized to the cell surface. The binding between growth factors and their cognate receptors mediates downstream pathways that play important roles in cell proliferation, migration, and differentiation.[2] Demand for low-cost and bioactive growth factors is expected to increase substantially as the nascent field of cellular agriculture continues to develop.[3–5] This new category of applications highlights the need for robust protocols capable of producing growth factors using tractable biological systems. A key challenge in producing many recombinant growth factors, which are of eukaryotic origin, is their requirement for the formation of disulfide bonds[6,7] which can make soluble expression in bacterial systems difficult.[8] This has resulted in a myriad of strategies for producing growth factors that range from expression in bacterial systems as inclusion bodies[9] to production using high-cost mammalian expression systems (e.g., Chinese Hamster Ovary cells).[10] To simplify growth factor production, we have developed a standardized workflow that allows for expression and purification of multiple families of growth factor including fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF) and transforming growth factor β1 (TGF-β1). The key variation in the proposed procedures for these respective growth factors is the choice of specific expression vector; additionally, some of the established protocols include additional optional downstream purification steps that were beneficial for specific growth factor families. We have validated that all four of these growth factor families are produced in soluble, bioactive forms using this workflow, but we have also used the same generalized procedure for producing additional growth factor families including epidermal growth factor (EGF), leukemia inhibitory factor (LIF) and myostatin. Consequently, we anticipate that this protocol will be suitable for bacterial expression of a wide array of growth factors and other disulfide-rich proteins.

This protocol outlines the end-to-end process for expressing and purifying the bioactive growth factors. This first phase of the protocol focuses on construct design, cloning, and expression testing to identify the most suitable combination of expression vector and host bacterial strain for producing growth factors of interest. The second phase outline the steps for large-scale expression and purification. Finally, the third stage covers strategies for validating the biological activity and usage of the growth factors.

Institutional permissions

Undertaking the protocol requires adherence to local institutional guidelines for laboratory safety and ethics. For bioactivity testing, the user will require access to Biosafety Level 2 (BSL2) facility for cell culture work.

Identifying growth factor genes and construct design

Timing: 1 day

1. Identify the coding sequence (DNA) or amino acid sequence for your growth factor of interest. These can be downloaded from GenBank or Uniprot. For this protocol, we use basic FGF (FGF2) as an example (UniProt ID: P09038).

   Note: This protocol has been successfully applied to obtain multiple orthologs of each growth factor family outlined above. Sequence diversity between orthologs was found to manifest as differences in the yield from recombinant expression under the same conditions and differences in the ability to support eukaryotic cell growth in culture. Therefore, it is recommended that more than one ortholog be tested to improve outcomes.

2. Design the construct for expression of the mature growth factor in the active form. For human FGF-2 this corresponds to the polypeptide encompassing the residues 135 to 288.

   Note: Many growth factors are initially produced as larger precursor proteins that are processed post-translationally into the mature, active growth factor. For this protocol, it is critical that the construct is designed to express only the mature, bioactive form of the growth factor. Sequences for the mature, bioactive forms of the growth factors described above can be found in Venkatesan, et al.[1], from product pages of commercial suppliers of growth factors or other general resources.

3. Codon optimize the coding sequence for the mature growth factor for expression in *Escherichia coli*.

   Note: Codon optimization services are available through IDT DNA (https://www.idtdna.com/pages/tools/codon-optimization-tool), Telesis Bio, Twist, BioBasic, and many other companies offering gene synthesis services.

4. Append the 30 base pair homology arm sequences to the 5′ and 3′ ends of the codon-optimized sequence. These homology arms are compatible with the expression vectors pMCSG53, pMCSG53-DsbC, pMCSG53-DsbA, and pMCSG53-GB1 (Figure 1).
   a. **5′ Homology Arm Sequence:** GGTACCGAGAACCTGTACTTCCAATCCAAT.
   b. **3′ Homology Arm Sequence:** ATTGGAAGTGGATAACGGATCCGAATTCGA.

   ⚠ CRITICAL: Do not add the homology arm sequences until after codon optimization. Changes to the sequences of the homology arms will render the constructs incompatible for downstream cloning into the expression vectors.

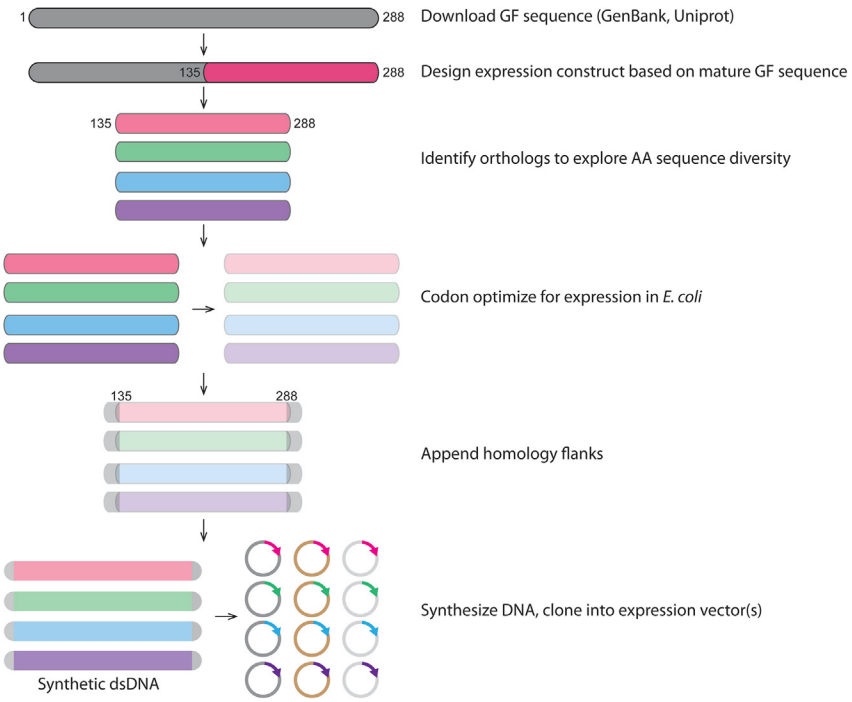

Figure 1. Construct design overview

Workflow for identifying growth factor sequences, curating a list of orthologs and creating expression constructs for screening.

5. Order synthetic, codon-optimized constructs for cloning.

   Note: These can be ordered through any of the aforementioned gene synthesis service providers. Upon arrival, it is recommended to resuspend the dsDNA constructs in nuclease-free water at a concentration of 25–100 ng/μL.

Cloning and transformation

Timing: 5 days

6. Acquire the appropriate expression vector for your growth factor of choice.

   Note: The specific expression vectors depend on your growth factor of interest and in some cases, it is advisable to test more than one expression vector. For FGF2, we use pMCSG53 for expression.

   a. **FGF1/FGF2.** Recommended vector: pMCSG53.[11]
   b. **IGF1/IGF2.** Recommended vector: pMCSG53-DsbC (Addgene: 186623).
   c. **PDGF.** Recommended vector: pET-His6-Thioredoxin-TEV-LIC (Addgene: 29712) or pMCSG53-DsbA (Addgene: 186624).
   d. **TGF-β1.** Recommended vector: pMCSG53-DsbC.
   e. Recommended boundaries of active domains of each growth factor are listed below:

Growth factor	Active domain
FGF1	16–155
FGF2	135–288
IGF1	49–118
IGF2	25–91
PDGF	82–190
TGF- β1	279–390

Note: The exact domain boundaries will differ depending on which ortholog is selected for purification. It is recommended that users carefully examine the desired sequence to ensure the correct active domain boundaries have been selected prior to proceeding further.

Note: The pMCSG53, pMCSG53-DsbC and pMCSG53-DsbA vectors contain a ccdB insert that allow for negative selection. They require a ccdB survival strain of *E. coli* for propagation.

7. Digest the vector using SspI-HF (NEB) for linearization and to remove the ccdB insert.
   a. Set up the reaction as follows:

Component	Amount
Plasmid Vector	1 μg
SspI-HF	1 μL
10X rCutSmart Buffer	2 μL
Nuclease-free H_2O	Up to 20 μL

   b. Incubate the reaction for 1 h at 37°C.

Note: The manufacturer suggests an incubation time of 15 min for digestion with this enzyme; however, in our experience optimal results are achieved using a 1 h digestion time.

   c. After incubation, add 1 μL of Quick CIP (calf intestinal alkaline phosphatase) (NEB) to the reaction mix and incubate for 1 h at 37°C.
   d. Purify the digested DNA using a Zymo Research DNA Clean and Concentrator Kit.
   e. Quantify the DNA by measuring absorbance at 260 nm using a DeNovix DS-11 microvolume spectrophotometer (or equivalent), perform quality control measurements by reading the 260/280 and 260/230 ratios.
8. Insert the codon-optimized constructs into the appropriate expression vector using the Gibson Assembly HiFi 1-Step Kit (Telesis Bio).
   a. Set up the reaction as follows:

Component	Amount
Linearized plasmid vector	25 ng
Codon-optimized insert	20–25 ng
Nuclease-free H_2O	Up to 5 μL
Gibson Assembly master mix (2X)	5 μL

Note: Sample should be mixed vigorously prior to addition of Gibson Assembly master mix. For full manufacturer's instruction see: https://telesisbio.com/products/benchtop-reagents/gibson-assembly-kits/

   b. Incubate the reaction for 1 h at 50°C.

c. Dilute the reaction by adding 20 μL of nuclease-free water. Use immediately for transformation or store at −20°C.

9. Transform the Gibson Assembly product into DH5α chemically competent cells or an alternative *recA1*- strain of *E. coli* (e.g., Top10).

Note: The manufacturer of the Gibson Assembly HiFi 1-Step Kit recommends using *E. cloni* 10G *E. coli* (Lucigen) competent cells. We have encountered no issues using DH5α competent cells.

a. Thaw competent cells.
b. Add 2 μL of diluted Gibson Assembly product to ~50 μL of competent cells.
c. In parallel, in a separate tube, add 0.5–1 μL of linearized plasmid vector to ~50 μL of competent cells. This serves as a Vector Only Control (VOC).
d. Incubate at room temperature for 2 min.
e. Plate the entire contents onto a pre-warmed LB agar plate containing 100 μg/mL of Ampicillin, spread using sterile glass beads.

Note: When using ampicillin as a selectable marker, the standard incubation on ice, heat shock and out-growth steps are not required for transformation.

f. Invert plates and incubate at 37°C for 12–16 h.

10. Analyze growth on plates. There should be no growth on the VOC.
11. Pick colonies for colony screening.
    a. Using a sterile pipette tip, pick single colonies and resuspend them in 10 μL of sterile water in a PCR tube.

Note: We recommended picking 2 colonies for every 1000 bp of insert. Half will be used for colony screening (see step 12), while the other half can be stored at −20°C until further use.

12. Set up colony screening PCR reaction.
    a. Prepare the following reaction mixture.

Component	Amount
Forward primer (10 μM stock)	1 μL
Reverse primer (10 μM stock)	1 μL
Template (resuspended colony from step 11)	5 μL
Nuclease-free H_2O	3 μL
Quick-Load Taq 2X Master Mix	10 μL

i. Forward Primer Sequence (T7): TAATACGACTCACTATAGGG.
ii. Reverse Primer Sequence (T7-ter): GCTAGTTATTGCTCAGCGG.

    b. Perform the PCR reaction in C1000 Thermal Cycler (Bio-Rad) using the following cycling program:

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
TD Denaturation	95°C	30 s	
TD Annealing	55°C, −1°C /cycle	30 s	10
TD Extension	68°C	1 min	
Denaturation	95°C	30 s	
Annealing	45°C	30 s	25
Extension	68°C	1 min	
Final Extension	68°C	5 min	1
Hold	12°C	Indefinitely	

Note: TD refers to the touchdown gradient. Use of a touchdown gradient improves specificity during amplification.

c. Analyze colony screening PCR products via agarose gel electrophoresis to check that the amplicon is the correct size before proceeding.

Note: The expected amplicon size differs depending on which expression vector is being used. For FGF2, the expected amplicon size is ~770 bp. Any colonies that produced unexpected amplicon sizes should be discarded.

13. Use the remaining 5 μL of colony resuspension (from step 11a) to inoculate 3 mL of LB broth with ampicillin (100 μg/mL).
14. Incubate at 37°C in a shaking incubator at ~250 rpm for 12–16 h.
15. Purify the plasmids using a Presto Mini Plasmid Kit (Geneaid) according to the manufacturer's specifications found at https://www.geneaid.com/Plasmid-DNA-Purification/PDH
16. Send an aliquot of purified plasmid for DNA sequencing.
17. Store remaining plasmid at −20°C until future use.

Small-scale expression testing

Timing: 2–3 days

18. Transform the sequence-verified constructs into a selection of *E. coli* expression strains for screening. The strains selected depend on the growth factor family being expressed. We found success with BL21-Gold(DE3) (Agilent) and SHuffle T7 Express (NEB) for multiple growth factor families. For FGF2 we transform into BL21-Gold(DE3).
    a. Thaw chemically competent cells.
    b. Add 50 ng of FGF2 expression construct to ~50 μL of competent cells.
    c. Optional: Heat shock the cells by incubating them at 42°C for 45 s.
    d. Incubate at room temperature for 2 min.
    e. Plate the entire contents onto a pre-warmed LB agar plate containing 100 μg/mL of Ampicillin, spread using sterile glass beads.
    f. Incubate at 37°C for 12–16 h.
19. Pick 3–5 colonies using a sterile pipette tip and use them to inoculate 2 mL of LB broth containing ampicillin (100 μg/mL).

Note: If testing a small number of growth factors or growth factor orthologs, this can be done in a glass test tube or falcon tube. For larger sets (multiple orthologs or multiple growth factor families), this small-scale culturing can be performed in a 24-well deep well block.

Note: The specific antibiotics used in the LB broth will depend on the *E. coli* expression strain used. BL21-Gold(DE3) and SHuffle T7 Express do not require additional selection; however other strains such as Rosetta-gami 2 (Novagen) require chloramphenicol for maintenance of additional plasmids (e.g., pRARE2) that can aid in protein expression.

20. Incubate at 37°C in a shaking incubator at ~250 rpm. Grow until the cultures reach an optical density (OD_{600}) of between 0.6 – 0.8.
21. Reduce the temperature of the incubator to 20°C, allow cultures to cool.
22. While cultures are cooling, making 0.5 M IPTG by dissolving 0.238 *g* of IPTG in 2 mL of MilliQ ultrapure water. Sterilize using a 0.22 μm syringe filter.
23. Induce cultures by adding IPTG to a final concentration of 1 mM. For a 2 mL culture, add 4 μL of 0.5 M IPTG solution.
24. Incubate at 20°C in a shaking incubator at ~250 rpm for 16 h.

25. Transfer culture to a 1.7 mL microcentrifuge tube.
26. Harvest cells by centrifuging at 5000 × *g* at 4°C for 20 min in an Eppendorf 5425 R Microcentrifuge.

Note: Alternative non-refrigerated microcentrifuges can be used by placing them in a cold room or cold cabinet prior to centrifugation.

27. Carefully remove the supernatant using a P1000 pipette.
28. Resuspend the cell pellet in 100 μL of binding buffer [300 mM NaCl, 50 mM HEPES pH 7.5, 5 mM imidazole, 5% glycerol] by pipetting.
29. Add 1 μL of 100X protease inhibitor cocktail (PIC) [100 mM benzamidine HCl, 50 mM PMSF in ethanol].
30. Freeze samples by transferring to −80°C.

Pause Point: Samples can be stored at −80°C for up to one month.

31. Thaw samples on ice.
32. Add lysozyme to a final concentration of 1.25 mg/mL. This is achieved by adding 5 μL of 25 mg/mL lysozyme stock solution.
33. Incubate samples on ice for 30 min. At 5-min intervals, invert the tubes several times to avoid separation.
34. Freeze samples by transferring to −80°C.
35. Thaw samples on ice.
36. Repeat steps 34 & 35 two more times for a total of three freeze-thaw cycles after lysozyme digestion.
37. Transfer a 25 μL aliquot to a new 1.7 mL microcentrifuge tube labeled "**Total Lysate**".

Note: The sample can be very viscous at this point. If difficulties with sample transfer are encountered, add 1 μL of commercial DNase I.

38. Centrifuge the remaining (~75 μL) cell lysate at 20,000 × *g* for 25 min at 4°C.
39. Transfer the supernatant to a new 1.7 microcentrifuge tube labeled "**Clarified Lysate**".
40. Analyze 5 μL of the **Total Lysate** and **Clarified Lysate** via SDS-PAGE using a 15% Tris-Glycine Mini Gel (Figure 2).

Note: Protein expression should be unambiguous at this stage as this protocol is intended to produce high yields of growth factors. When analyzing the gel, be sure to account for the size of any fusion tags when assessing for protein expression. Your growth factor of interest should be present in the **Total Lysate** lane and the **Clarified Lysate** lane. The former is used to test for protein expression, while the latter is an indicator of protein solubility. Samples that show clear evidence of growth factor of interest in the **Clarified Lysate** should then proceed to large-scale purification. If screening multiple expression vectors and/or *E. coli* expression strains, this analysis can be used to make general comparisons as to which combination of vector and *E. coli* strain is most suitable for your specific growth factor.

TEV protease purification

Timing: 4 days

41. Transform TEV protease expression construct (pTEVSH – Addgene: 125194.) into BL21 (DE3) RIL competent cells.
    a. Thaw chemically competent BL21-CodonPlus (DE3)-RIL cells on ice.
    b. Thaw pTEVSH plasmid.

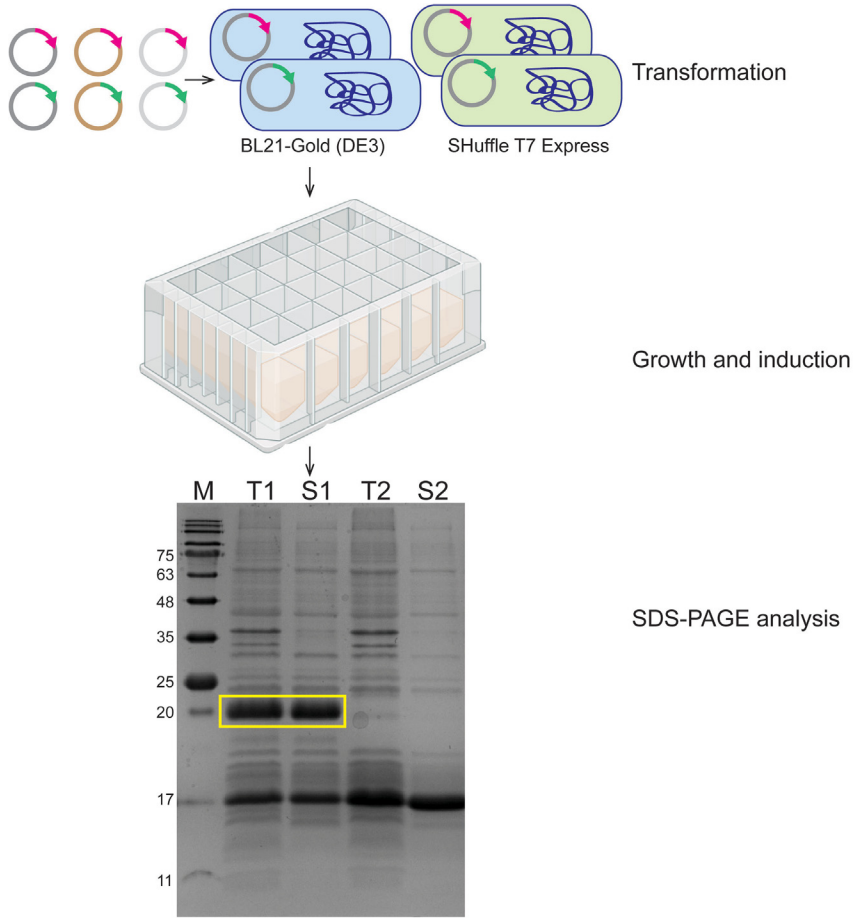

Figure 2. Expression testing overview

Expression constructs are transformed into *E. coli* expression strains. Growth and induction is followed by SDS-PAGE analysis. Representative results are shown: T1 – Total Lysate ortholog 1; S1 – Soluble Fraction ortholog 1; T2 – Total Lysate ortholog 2; S2 – Soluble Fraction ortholog 2. In this example, T1 is expressed and soluble (as indicated by demarcation in yellow) and suitable for large-scale protein expression. T2 is not expressed and would not be advanced to the large-scale expression step.

c. Mix 50 ng of pTEVSH plasmid with ~50 μL of competent cells.
d. Incubate at room temperature for 2 min.
e. Add 300 μL of LB broth to mixture.
f. Incubate in a shaking incubator at 37°C and ~250 rpm for 1 h.
g. Plate 100 μL onto a pre-warmed LB agar plate containing 100 μg/mL of Ampicillin and 25 μg/mL of chloramphenicol, spread using sterile glass beads.
h. Incubate at 37°C for 12–16 h.

42. Inoculate and grow the overnight starter culture.
    a. Pick 3–5 colonies from the plate using a sterile pipette tip.
    b. Use to inoculate 25 mL of LB broth containing 100 μg/mL of ampicillin and 25 μg/mL of chloramphenicol in a 250 mL Erlenmeyer flask.
    c. Grow overnight in a shaking incubator at 37°C and ~220 rpm for 12–16 h.
43. Inoculate the large-scale cultures (2 × 1L) by diluting the overnight starter culture 1:100 (v/v) into 1 L of pre-warmed (to 37°C) LB broth containing 100 μg/mL of ampicillin in a 4L Erlenmeyer flask.
44. Grow the cells in a shaking incubator at 37 °C at ~220 rpm until the cultures reach an optical density (OD_{600}) of 0.6–0.8.

45. Lower the temperature of the shaking incubator to 20°C and allow the cultures to cool.
46. Induce the cultures by adding IPTG to a final concentration of 1 mM.
47. Incubate cultures in the shaking incubator at 20°C and ~220 rpm for 16 h (overnight).
48. Transfer cultures to 1L centrifuge bottles (Beckman).

⚠ CRITICAL: Centrifuge bottles must be balanced prior to centrifugation or there is a risk of damage/injury. Sterile water can be used to balance at this stage.

49. Harvest cells by centrifugation at 5000 × *g* for 20 min at 4°C.
50. Carefully remove the supernatant.
51. Resuspend cell pellets using 20–25 mL of binding buffer per liter of culture.

Pause Point: Resuspended cell pellets can be stored at −80°C for up to one month.

52. Lyse the resuspended cells using a QSonica Ultrasonicator (or equivalent). Amplitude should be set to ~65% but depends on the specific power supply.
    a. The samples are placed on ice and sonication proceeds in 3-s bursts followed by 6-s rests.
    b. Continue process for 10–15 min of **sonication time**. The total time for this step will be ~30–45 min.
53. Centrifuge the lysate at 20,000 × *g* and 4°C for 45 min to remove cell debris and insoluble material.
54. During centrifugation (step 15), equilibrate 0.5–1 mL of Ni-NTA resin per liter of culture in binding buffer using a Bio-Rad gravity flow column.
55. Collect supernatant after centrifugation, add 0.5–1 mL of Ni-NTA equilibrated in binding buffer in a 50 mL falcon tube.
56. Incubate the supernatant and Ni-NTA at 4°C under gentle agitation for 2 h for batch binding.
57. Centrifuge the samples using a Beckman Coulter Allegra X-14R Centrifuge at 200 × *g* for 5 min at 4°C.
58. Carefully remove supernatant. Be sure not to disturb the Ni-NTA pellet. Users may choose to keep an aliquot of the supernatant for analysis via SDS-PAGE; however, in our experience this is not required.
59. Add 25 mL of wash buffer.
60. Invert tube several times to resuspend Ni-NTA pellet.
61. Centrifuge the samples using a Beckman Coulter Allegra X-14R Centrifuge at 200 × *g* for 5 min at 4°C.
62. Repeat steps 58–61 two more times.
63. Add 25 mL of wash buffer and resuspend the Ni-NTA pellet by inverting the tube.
64. Transfer resuspension into a Bio-Rad gravity flow column.
65. Monitor the protein content in the column flowthrough by mixing 10 μL with 200 μL of Coomassie Plus Bradford Reagent (Thermo Fisher).
66. Continue washing the Ni-NTA bed with wash buffer until no protein is present in the column flowthrough (no or minimal color change when mixed with Bradford reagent).
67. Stopper the column.
68. Add 10 mL of **TEV Elution Buffer** [100 mM NaCl, 50 mM Tris pH 7.5, 20% glycerol, 250 mM imidazole].
69. Remove the stopper and collect the flowthrough in a 15 mL falcon tube.
70. Using a DeNovix DS-11 microvolume spectrophotometer (or equivalent), measure the absorbance of the sample at 280 nm. Calculate the protein concentration using the Beer-Lambert law.

Note: The TEV protease concentration should be approximately 3–4 mg/mL at this stage, which is the target concentration.

71. Analyze the preparation of TEV protease via SDS-PAGE by running 10 μL of purified sample on a 12% Tris-Glycine mini gel. TEV protease is 33.4 kDa.
72. Dispense the purified TEV protease into 500 μL aliquots in 1.7 mL Eppendorf tubes.

73. Flash freeze using liquid nitrogen.
74. Store at −80°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Phospho-p44/42 MAPK ERK1/2 rabbit mAb (1:1000)	Cell Signaling Technology	cat. no. 9101
P44/42 MAPK ERK1/2 rabbit mAb (1:1000)	Cell Signaling Technology	cat. no. 4695
Phospho-smad2-rabbit mAb (1:1000)	Cell Signaling Technology	cat. no. 3108
Smad2 rabbit mAb (1:1000)	Cell Signaling Technology	cat. no. 5339
Bacterial and virus strains		
E. coli DH5α	CGSC	CGSC# 14231
E. coli BL21-Gold (DE3) Competent Cells	Agilent	cat. no. 230132
E. coli SHuffle T7 Express Competent Cells	NEB	cat. no. C3029J
E. coli BL21-CodonPlus (DE3)-RIL Competent Cells	Agilent	cat. no. 230245
Chemicals, peptides, and recombinant proteins		
Tryptone (bacteriological)	BioShop	cat. no. TRP402
Yeast extract	BioShop	cat. no. YEX401
Sodium chloride	VWR	cat. no. BDH9286
Agar, bacteriological grade	BioShop	cat. no. AGR001
Ampicillin, sodium salt	BioShop	cat. no. AMP201
Chloramphenicol	BioShop	cat. no. CLR201
HEPES, biotechnology grade	BioShop	cat. no. HEP001
Potassium chloride	BioShop	cat. no. POC888
Potassium phosphate monobasic	BioShop	cat. no. PPM666
Sodium phosphate dibasic	BioShop	cat. no. SPD600
TRIS (base), UltraPure	BioShop	cat. no. TRS001
Imidazole	Sigma	cat. no. 15513
Glycerol	BioShop	cat. no. GLY001
HisPur Ni-NTA resin	Thermo Fisher	cat. no. 88221
30% Acrylamide/Bis solution, 37.5:1	Bio-Rad	cat. no. 1610159
TEMED	Bio-Rad	cat. no. 1610800
Ammonium persulfate, electrophoresis grade	BioShop	cat. no. AMP001
IPTG	GoldBio	cat. no. I2481C100
DMEM	Gibco	cat. no. 11995-065
Fetal bovine serum	Gibco	cat. no. 10100-147
1X RIPA Buffer	Cell Signaling Technology	cat. no. 9806
SspI-HF	NEB	cat. no. R3132L
Quick-Load Taq 2X Master Mix	NEB	cat. no. M0271L
rCutSmart Buffer	NEB	cat. no. R3132L
Tobacco Etch Virus (TEV) protease	This study	N/A
FGF2	This study	N/A
Critical commercial assays		
Gibson Assembly HiFi	Telesis Bio	cat. no. GA1100
Coomassie Plus (Bradford) Protein Assay	Thermo Fisher	cat. no. 23236
Clarity Western ECL	Bio-Rad	cat. no. 170-5060
Experimental models: Cell lines		
NIH-3T3 Mouse fibroblasts	ATCC	cat. no. CRL-1658
Oligonucleotides		
T7, promoter TAATACGACTCACTATAGGG	IDT DNA	N/A
T7, terminator GCTAGTTATTGCTCAGCGG	IDT DNA	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pMCSG53		N/A
pMCSG53-DsbA	Addgene	cat. no. 186624
pMCSG53-DsbC	Addgene	cat. no. 186623
TEVSH	Addgene	cat. no. 125194
pET-TrxA	Addgene	cat. no. 29712
Software and algorithms		
SnapGene Viewer	SnapGene	Snapgene.com
Other		
Glass Econo-Columns	Bio-Rad	cat. no. 7376116
SnakeSkin dialysis tubing, 10K	Thermo Fisher	cat. no. 68100
SnakeSkin dialysis tubing, 3.5K	Thermo Fisher	cat. no. 88242
Dialysis clips	Thermo Fisher	cat. no. 68011
Float buoys	Thermo Fisher	cat. no. 66430

MATERIALS AND EQUIPMENT

Binding buffer		
Reagent	Final concentration	Amount
5M NaCl	300 mM	30 mL
1M HEPES pH 7.5	50 mM	25 mL
2M Imidazole	5 mM	1.25 mL
Glycerol (50% v/v)	5%	50 mL
ddH_2O	N/A	393.75 mL
Total	N/A	500 mL
Store at 4°C for up to one month.		

Wash buffer		
Reagent	Final concentration	Amount
5M NaCl	300 mM	60 mL
1M HEPES pH 7.5	50 mM	50 mL
2M Imidazole	30 mM	30 mL
Glycerol (50% v/v)	5%	100 mL
ddH_2O	N/A	760 mL
Total	N/A	1000 mL
Store at 4°C for up to one month.		

Elution buffer		
Reagent	Final concentration	Amount
5M NaCl	300 mM	6 mL
1M HEPES pH 7.5	50 mM	5 mL
2M Imidazole	250 mM	12.5 mL
Glycerol (50% v/v)	5%	10 mL
ddH_2O	N/A	66.5 mL
Total	N/A	100 mL
Store at 4°C for up to one month.		

Dialysis buffer		
Reagent	Final concentration	Amount
5M NaCl	300 mM	60 mL
1M HEPES pH 7.5	50 mM	50 mL
Glycerol (50% v/v)	5%	100 mL
ddH_2O	N/A	790 mL
Total	N/A	1000 mL
Store at 4°C for up to one month.		

Ampicillin stock solution		
Reagent	Final concentration	Amount
Ampicillin sodium salt	100 mg/mL	1 *g*
ddH_2O	N/A	To 10 mL
Total	N/A	10 mL
Store at −20°C for up to one year.		

Note: Prior to use or storage, the solution should be sterilized using a 0.22 μm syringe filter.

1M IPTG		
Reagent	Final concentration	Amount
IPTG	1 M	2.38 *g*
ddH_2O	N/A	To 10 mL
Total	N/A	10 mL
Store at −20°C for up to one year.		

Note: Prior to use or storage, the solution should be sterilized using a 0.22 μm syringe filter.

100X protease inhibitor cocktail (PIC)		
Reagent	Final concentration	Amount
Benzamidine HCl	100 mM	1.56 *g*
PMSF	50 mM	0.87 *g*
95% Ethanol	N/A	To 100 mL
Total	N/A	100 mL
Store at −20°C for up to one month.		

LB broth		
Reagent	Final concentration	Amount
Tryptone	1%	10 *g*
Yeast Extract	0.5%	5 *g*
NaCl	1%	10 *g*
ddH_2O	N/A	To 1000 mL
Total	N/A	1000 mL
Store at room temperature until use.		

Note: LB broth must be autoclaved and allowed to cool prior to use.

Alternatives: For cellular agriculture application it may be advisable to use vegetable tryptone to avoid the use of animal-derived components

LB Agar with ampicillin		
Reagent	Final concentration	Amount
Tryptone	1%	5 *g*
Yeast Extract	0.5%	2.5 *g*
NaCl	1%	5 *g*
Agar	1.5%	7.5 *g*
Ampicillin stock (100 mg/mL)*	100 μg/mL	0.5 mL
ddH_2O	N/A	To 500 mL
Total	N/A	500 mL
Store at 4°C for up to one month.		

⚠ CRITICAL: Ampicillin stock is not added until after the LB agar has been autoclaved and allowed to cool to ~ 50°C. While adding ampicillin, proper aseptic technique should be practiced and LB agar with ampicillin should be poured in petri dish plates immediately after.

Alternatives: For cellular agriculture application it may be advisable to use vegetable tryptone to avoid the use of animal-derived components.

1X PBS, pH 7.4		
Reagent	Final concentration	Amount
NaCl	137 mM	8 *g*
KCl	2.7 mM	0.2 *g*
Na_2HPO_4	10 mM	1.44 *g*
KH_2PO_4	1.8 mM	0.24 *g*
ddH_2O	N/A	To 1000 mL
Total	N/A	1000 mL
Store at 22°C for up to one month.		

Note: When making 1X PBS, add water to a final volume of approximately 900 mL, then adjust the pH to 7.4 using concentrate HCl, then adjust the final volume to 1000 mL.

⚠ CRITICAL: Concentrate HCl is hazardous and can cause injury. Proper personal protective equipment is required, and a fume hood should be used when working with HCl.

Alternatives: Commercial 1X PBS can be purchased from a variety of suppliers and used as an alternative.

STEP-BY-STEP METHOD DETAILS

Transformation and large-scale protein expression

Timing: 4 days

This step encompasses the transformation of the growth factor expression construct (pMCSG53-FGF2, in this specific case) into the best performing *E. coli* expression strain as identified in the small-scale expression testing (Figure 3). After transformation, cells are grown up as part of a large-scale culture, induced with IPTG to express the growth factor protein of interest and the cells are collected for downstream protein purification.

1. Transform expression construct (pMCSG53-FGF2) into BL21-Gold (DE3).
   a. Thaw chemically competent BL21-Gold (DE3) cells on ice.
   b. Thaw pMCSG53-FGF2 plasmid.
   c. Mix 50 ng of pMCSG53-FGF2 plasmid with ~50 μL of competent cells.

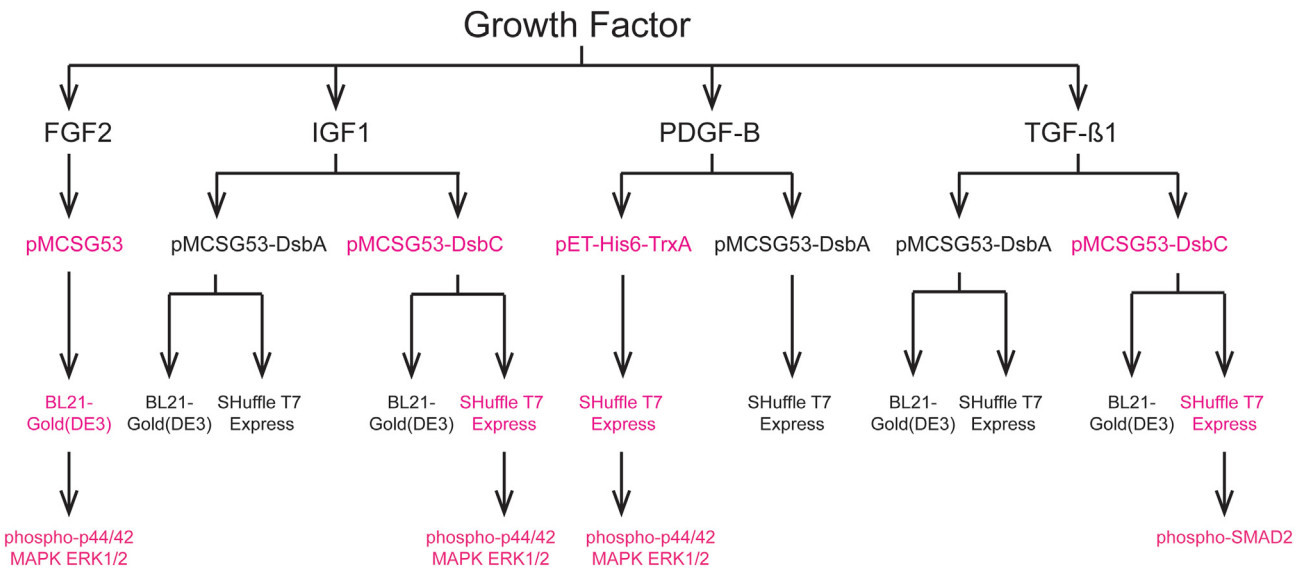

Figure 3. Design flowchart for selected growth factor families

Recommended expression vectors and *E. coli* strains for each growth factor family, based on our previous results, are colored in magenta.

d. Incubate at room temperature for 2 min.
e. Plate the entire contents onto a pre-warmed LB agar plate containing 100 μg/mL of Ampicillin, spread using sterile glass beads.
f. Incubate at 37°C for 12–16 h.

2. Inoculate and grow the overnight starter culture.
   a. Pick 3–5 colonies from the plate using a sterile pipette tip.
   b. Use to inoculate 25 mL of LB broth containing 100 μg/mL of ampicillin in a 250 mL Erlenmeyer flask.
   c. Grow overnight in a shaking incubator at 37°C and ~220 rpm for 12–16 h.

Note: The size of this overnight starter culture depends on the large-scale culture volume. Using this protocol, we have achieved yields in the mg/L range for all growth factor families tested. Depending on the needs of the end-user, the scope of the large-scale purification could range from 1L to 6L (6 × 1L cultures). We recommend diluting the overnight starter culture 1:100 for large-scale growth. Consequently, the size of the starter culture should be adjusted to ensure a sufficient amount is available for inoculation.

3. Inoculate the large-scale cultures (2 × 1L) by diluting the overnight starter culture 1:100 (v/v) into 1 L of pre-warmed (to 37°C) LB broth containing 100 μg/mL of ampicillin in a 4L Erlenmeyer flask.

Note: We typically grow 2 L (2 × 1L) for purification. Using even numbers of flasks allows for easy balance of the shaking incubator and ensures balance during downstream centrifugation steps.

4. Grow the cells in a shaking incubator at 37°C at ~220 rpm until the cultures reach an optical density (OD_{600}) of 0.6–0.8.
5. Lower the temperature of the shaking incubator to 20°C and allow the cultures to cool.
6. Induce the cultures by adding IPTG to a final concentration of 1 mM.
7. Incubate cultures in the shaking incubator at 20°C and ~220 rpm for 16 h (overnight).
8. Transfer cultures to 1L centrifuge bottles (Beckman).

⚠ CRITICAL: Centrifuge bottles must be balanced prior to centrifugation or there is a risk of damage/injury. Sterile water can be used to balance at this stage.

9. Harvest cells by centrifugation at 5000 × *g* for 20 min at 4°C.
10. Carefully remove the supernatant.
11. Resuspend cell pellets using 20–25 mL of binding buffer per liter of culture.
12. Add 200–250 μL of 100X PIC.

Pause Point: Resuspended cell pellets can be stored at −80°C for up to one month.

Growth factor purification

Timing: 2–3 days

This step outlines purification of the growth factors, removal of the affinity tag/fusion partner, and gel filtration to further improve the purity and to facilitate buffer exchange into the desired storage buffer.

13. Lyse the resuspended cells using a QSonica Ultrasonicator (or equivalent). Amplitude should be set to ~65% but depends on the specific power supply.
    a. The samples are placed on ice and sonication proceeds in 3-s bursts followed by 6-s rests.
    b. Continue process for 10–15 min of **sonication time**. The total time for this step will be ~30–45 min.

⚠ CRITICAL: Follow the manufacturer's guidelines with respect to power settings for your specific sonicator probe. Failure to follow these guidelines can result in damage to the probe.

⚠ CRITICAL: The samples should be kept cool throughout the entire sonication process. This will require replenishment of the ice bath.

⚠ CRITICAL: Ensure that the sonicator probe is submerged, but not touching the walls of the vessel. If the probe is too close to the surface of your sample, it will result in foaming which can denature proteins.

Alternatives: Lysis can also be performed using a homogenizer or with a lysozyme and freeze-thaw approach

14. Take a small aliquot of the sonicated sample (10–15 μL) and label it **Total Lysate**. Store at −20°C until SDS-PAGE analysis.
15. Centrifuge the lysate at 20,000 × *g* and 4°C for 45 min to remove cell debris and insoluble material.
16. During centrifugation (step 15), equilibrate 0.5–1 mL of Ni-NTA resin per liter of culture in binding buffer using a Bio-Rad gravity flow column.
17. Collect supernatant after centrifugation. Take a small aliquot and label it **Clarified Lysate**. Store at −20°C until SDS-PAGE analysis.
18. To the remaining supernatant, add 0.5–1 mL of Ni-NTA equilibrated in binding buffer in a 50 mL falcon tube.
19. Add 100X PIC to the sample.
20. Incubate the supernatant and Ni-NTA at 4°C under gentle agitation for 1 h for batch binding.
21. Centrifuge the samples using a Beckman Coulter Allegra X-14R Centrifuge at 200 × *g* for 5 min at 4°C.

22. Carefully remove supernatant. Be sure not to disturb the Ni-NTA pellet. Users may choose to keep an aliquot of the supernatant for analysis via SDS-PAGE, particularly during the initial purification of a specific growth factor.
23. Add 25 mL of wash buffer.
24. Invert tube several times to resuspend Ni-NTA pellet.
25. Centrifuge the samples using a Beckman Coulter Allegra X-14R Centrifuge at 200 × *g* for 5 min at 4°C.
26. Repeat steps 22–25 two more times.
27. Add 25 mL of wash buffer and resuspend the Ni-NTA pellet by inverting the tube.
28. Transfer resuspension into a Bio-Rad gravity flow column.
29. Monitor the protein content in the column flowthrough by mixing 10 μL with 200 μL of Coomassie Plus Bradford Reagent (Thermo Fisher).

Note: The Bradford reagent will turn blue when protein is present. The speed of color change and intensity of the blue color can be used as visual markers to estimate how much protein is present.

30. Continue washing the Ni-NTA bed with wash buffer until no protein is present in the column flowthrough (no or minimal color change when mixed with Bradford reagent).
31. Stopper the column.
32. Add 10 mL of elution buffer.
33. Remove the stopper and collect the flowthrough in a 15 mL falcon tube.
34. Monitor the protein content in the column flowthrough by mixing 10 μL with 200 μL of Coomassie Plus Bradford Reagent (Thermo Fisher).
35. Continue adding elution buffer until no protein is present in the column flowthrough.
36. Take an aliquot of the eluted sample and label it **Elution**. Store at −20°C until SDS-PAGE analysis.
37. Using a DeNovix DS-11 microvolume spectrophotometer (or equivalent), measure the absorbance of the sample at 280 nm. Calculate the protein concentration using the Beer-Lambert law.
38. For removal of affinity tag and any fusion partners (e.g., if using pMCSG53-DsbC), add TEV protease to the eluted sample.
    a. To determine how much TEV protease to add use the following formula: 45 μg of TEV protease for every mg of protein.

Note: At this stage, for the purposes of TEV protease calculations, assume that 100% of the protein content present in the eluted sample is the growth factor protein of interest.

39. Prepare dialysis tubing and add sample containing TEV protease.
    a. Cut a 10–15 cm length of Snakeskin Dialysis Tubing, 10K mwco (Thermo Fisher).
    b. Clip the bottom shut using 2 Dialysis Tubing Clips (Thermo Fisher) facing opposite directions.
    c. Gently pour the sample into the open end of the dialysis tubing.
    d. Using your fingers, flatten the top portion of the dialysis tubing to remove air.
    e. Close the top of the tube using a single Dialysis Tubing Clip.
    f. Attach a Slide-A-Lyzer Float Buoy to the top (single) dialysis clip.

Note: At this stage, if purifying multiple orthologs of a growth factor or multiple growth factors, attach tape to the top of the float buoy to mark samples for identification.

40. Place dialysis tubing into a 1 L glass beaker containing pre-chilled dialysis buffer.
41. Add a magnetic stir bar and place the beaker on a stir plate in a cold room or cold cabinet.
42. Set the stir plate to gently stir (~25% of max settings depending on the specific stir plate used).

43. Incubate sample overnight (~16 h) at 4°C. During this stage TEV protease cleavage and dialysis will proceed concurrently.

Note: Users may choose to replace the dialysis buffer with freshly prepared buffer halfway through incubation but this was not required in our application of this protocol.

44. Remove the dialysis tubing from the beaker and carefully remove the float buoy and the top Dialysis Tubing Clip.
45. Carefully pour the sample from the tubing into a new 15 mL falcon tube. Be sure to squeeze the tubing with your fingers to ensure all liquid is transferred.
46. Pour sample over a Ni-NTA bed pre-washed with binding buffer in a stoppered Bio-Rad gravity flow column.

Note: The Ni-NTA from the previous chromatography step can be re-used for this step. If re-using, it is important to wash it with 20–30 mL of binding buffer before use.

47. Remove the stopper and collect the flowthrough.

Note: Upon TEV protease-mediated removal of the 6His affinity tag, the growth factor protein should no longer bind to the Ni-NTA column. Other impurities, as well as the TEV protease (which is 6His-tagged) should be retained by the Ni-NTA matrix.

48. After all flowthrough has been collected, re-apply the sample to the stoppered Ni-NTA matrix.
49. Remove the stopper and collect the flowthrough.
50. Repeat steps 48 and 49 one additional time.
51. Monitor the protein content in the last drops of column flowthrough by mixing 10 μL with 200 μL of Coomassie Plus Bradford Reagent (Thermo Fisher).
52. If protein remains in the flowthrough, add 1–2 mL of additional binding buffer and re-check using Bradford Reagent. Continue process until protein is no longer present in the flowthrough.
53. Label sample **2nd Ni-NTA Flowthrough** and store on ice.
54. Add 10 mL of wash buffer to the Ni-NTA matrix and collect flowthrough.
55. Label sample **2nd Ni-NTA Wash** and store on ice.

Note: In rare cases, some proteins inherently bind to Ni-NTA even in the absence of a 6His tag. Washing the Ni-NTA matrix with wash buffer and collecting will recover most proteins should this scenario arise.

56. Add 10 mL of elution buffer to the Ni-NTA matrix and collect flowthrough.
57. Label sample **2nd Ni-NTA EB** and store on ice.

Note: Washing the Ni-NTA matrix with elution buffer accounts for any potential issues with TEV protease cleavage.

58. Analyze 5 μL of the **Total Lysate** and **Clarified Lysate**, and 10 μL of Elution, **2nd Ni-NTA Flow-through**, **2nd Ni-NTA Wash** and **2nd Ni-NTA EB** via SDS-PAGE using a 15% Tris-Glycine Mini Gel.

Note: At this point, FGF2 should be present in the **2nd Ni-NTA Flowthrough** and the purity is expected to be approximately 90–95%. There should also be a characteristic downward shift when comparing the dominant band present in **Elution** and the dominant band in the **2nd Ni-NTA Flowthrough** sample. This shift indicates successful TEV protease cleavage and removal of the 6His and TEV sequence.

Note: At this point, the growth factors will be suitable for most downstream applications. The necessity of the remaining steps will depend on the end-user and their specific applications.

59. Transfer the purified growth factor sample (i.e., **2nd Ni-NTA Flowthrough**) to a 15-mL, 10K MWCO Amicon Ultra Centrifugal Filter.

Note: The filter pore size selected may differ depending on the growth factor target being purified. For example, EGF is smaller than 10K and should be concentrated using a 3K MWCO filter.

60. Centrifuge the samples using a Beckman Coulter Allegra X-14R Centrifuge at 3700 × *g* for 5 min at 4°C.
61. Gently mix the liquid remaining in the filter using a P200 pipette. Visually inspect for the presence of white precipitate. If no precipitate is present, continue to next step.
62. Centrifuge the samples using a Beckman Coulter Allegra X-14R Centrifuge at 3700 × *g* for 5 min at 4°C.
63. Continue performing steps 60–62 until the sample volume is reduced to approximately 0.5 mL or reaches a concentration of 10 mg/mL.

Note: We have routinely concentrated purified FGF2 orthologs to concentrations exceeding 10 mg/mL. Protein stability at high concentrations varies depending on the specific growth factor family.

64. Load the sample onto a Superdex 75 10/300 GL column pre-equilibrated with 1X PBS (or alternative buffer selected by end-user).
65. Run FPLC at a rate of 0.5 mL/min, set fraction volume to 0.5 mL.
66. Analyze 5 µL of fractions corresponding to peaks (as indicated by UV_{280} measurement) via SDS-PAGE using a 15% Tris-Glycine Mini Gel.
67. Combine fractions containing pure FGF2.
68. Using a DeNovix DS-11 microvolume spectrophotometer (or equivalent), measure the absorbance of the sample at 280 nm. Calculate the protein concentration using the Beer-Lambert law.
69. Adjust concentration to desired level either by diluting using 1X PBS or concentrating as described above in steps 60–62.
70. Aliquot samples and flash-freeze using liquid nitrogen.
71. Store at −80°C (Figure 4).

Western blotting to validate bioactivity

Timing: 2–4 days

This step outline one possible procedure for validating the bioactivity of purified growth factors. Validation via Western blotting seeks to show that the appropriate downstream pathways are being activated in response to treatment with growth factors. This step can also be used to compare different growth factor orthologs to determine which is most suitable for the desired applications of the end-user.

⚠ CRITICAL: These steps must be conducted in lamellar flow cabinet using proper aseptic technique.

72. Seed NIH-3T3 Murine fibroblasts (ATCC CRL-1658) into 6-well tissue culture plates at a density of 100,000 cells/well.

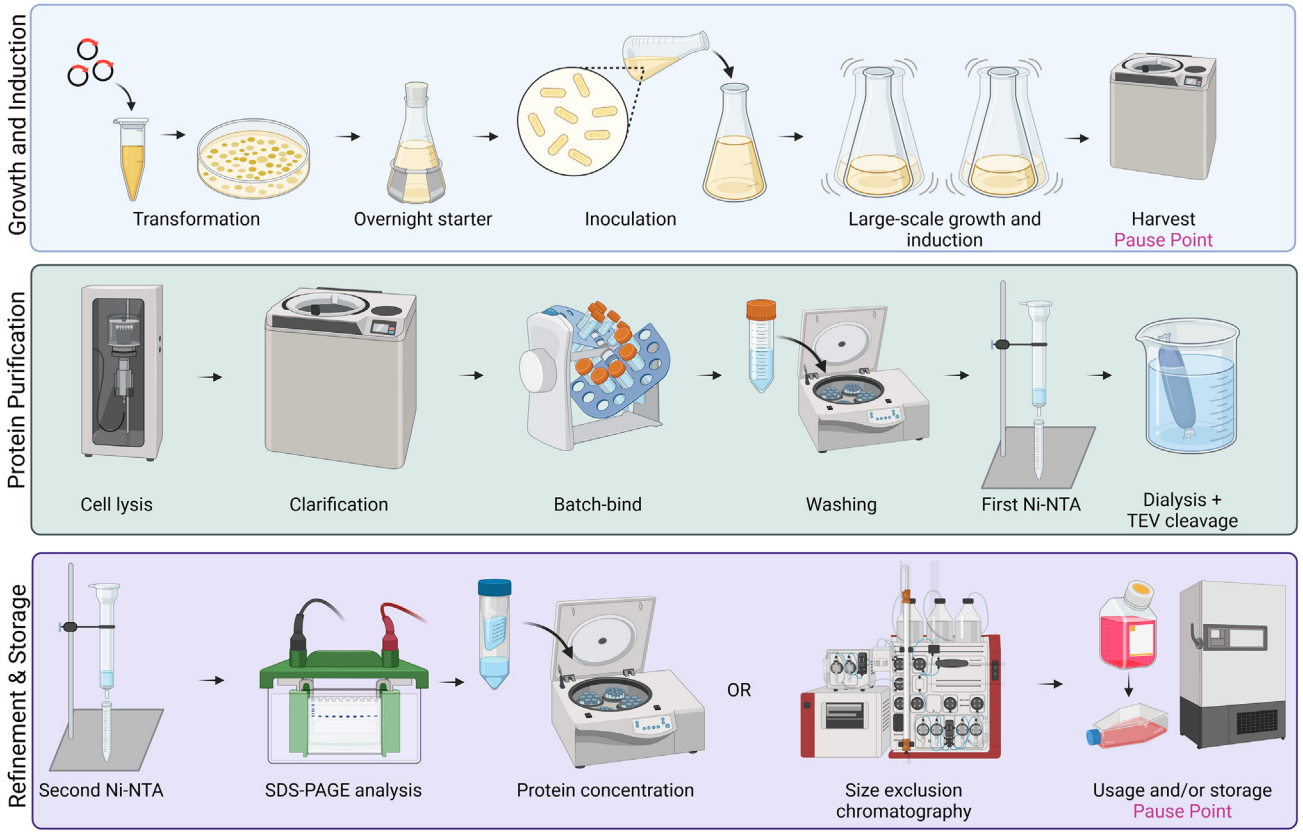

Figure 4. Schematic of the protocol for large-scale growth factor expression and purification

Note: A minimum of two wells is required to test a single growth factor (one for treated, one as a control well). Scale the number of wells accordingly depending on the number of growth factors, orthologs, or replicates desired. If testing for dose-dependency, additional wells will be required.

73. Add 1 mL of DMEM [high glucose, pyruvate] (GIBCO 11995065) containing 10% heat-inactivated fetal bovine serum to each well.
74. Move 6-well plate to a VWR Symphony CO_2 Incubator.
75. Incubate cells at 37°C and 5% CO_2 for 24 h to allow for attachment.
76. Move 6-well plate to lamellar flow cabinet.
77. Gently aspirate the media using a pipette, discard.
78. Wash cells by adding 1 mL of sterile 1X PBS.
79. Gently aspirate the 1X PBS using a pipette, discard.
80. Repeat steps 78 and 79.
81. Add 1 mL of DMEM [high glucose, pyruvate] containing 0.11% heat-inactivated fetal bovine serum to each well.
82. Move 6-well plate to a VWR Symphony CO_2 Incubator.
83. Incubate cells at 37°C and 5% CO_2 for 24 h for serum starvation.

Note: If using a cells/cell line grown in serum free conditions, the equivalent starvation step would be incubation in the absence of any growth factors typically used in growth media.

84. Move 6-well plate to lamellar flow cabinet.

85. Gently aspirate the media using a pipette, discard.
86. Wash cells by adding 1 mL of sterile 1X PBS.
87. Gently aspirate the 1X PBS using a pipette, discard.
88. Repeat steps 86 and 87.
89. Add 1 mL of DMEM [high glucose, pyruvate] containing 10 ng/mL of purified growth factor.

Note: The optimal concentration of purified growth factor will vary depending on which growth factor family is being tested. It is recommended to test a range of concentrations to determine the most suitable usage levels for the specific cell system of interest. Recommended starting concentrations are listed in the table below:

Growth factor	Recommended concentration
FGF2	10 ng/mL
IGF1	50 ng/mL
PDGF-BB	10 ng/mL
TGF-β1	1 ng/mL

90. Move 6-well plate to lamellar flow cabinet.
91. Incubate cells at 37°C and 5% CO_2 for 24 h.

⚠ CRITICAL: The incubation time for step 92 is dependent on the growth factor family being tested. For FGF2, PDGF-BB, and IGF1 we incubate for 24 h. If testing TGF-β1, cells were incubated for 45 min.

92. Move 6-well plate to lamellar flow cabinet.
93. Gently aspirate the media using a pipette.
94. Wash cells by adding 1 mL of sterile, cold 1X PBS.
95. Gently aspirate the media using a pipette.
96. Repeat steps 94 and 95.
97. Add 200 μL of ice-cold 1X RIPA buffer (Cell Signaling Tech. 9806) to each well.
98. Incubate at 4°C for 1 h to allow for cell lysis to occur.
99. Aspirate the liquid and transfer to a 1.7 mL microcentrifuge tube.
100. Centrifuge samples at 18,000 × *g* for 20 min at 4°C.
101. Remove supernatant and transfer to a new 1.7 mL microcentrifuge tube.
102. Add an equal volume of 2X Laemmli sample buffer.
103. Incubate at 95°C for 10 min.
104. Analyze 5–10 μL via SDS-PAGE using a 15% Tris-Glycine Mini Gel.

⚠ CRITICAL: Do note stain the Mini Gel prior to transfer to nitrocellulose.

105. Transfer to nitrocellulose using a Bio-Rad TransBlot Turbo Transfer System.
106. Place the nitrocellulose membrane in a small container containing blocking buffer [Tris-buffer saline (TBS) with 5% w/v BSA and 0.1% v/v Tween-20] and incubate under gentle agitation for 1 h.
107. Decant the blocking buffer into a waste container.
108. Add 10 mL of TBST [TBS and 0.1% v/v Tween-20] to container containing nitrocellulose membrane and incubate under gentle agitation for 10 min.
109. Decant the TBST solution into a waste container.
110. Repeat steps 108 and 109 two more times.
111. Dilute primary antibody 1:1000 in TBST + 5% BSA, add to nitrocellulose membrane.

Note: The choice of primary antibody will depend on the specific growth factor being tested. FGF2, IGF1 and PDGF-BB activate the MAPK Erk1/2 pathway, TGF-β1 activates SMAD signaling).

a. FGF2, IGF1, PDGF-BB: phospho-p44/42 MAPK ERK1/2 rabbit mAB (Cell Signaling Technologies 9101) and p44/42 MAPK ERK1/2 rabbit mAB (Cell Signaling Technologies 4695).
b. TGF-β1: phosphor-smad2 rabbit mAB (Cell Signaling Technologies 3108) and smad2 rabbit mAB (Cell Signaling Technologies 5339).

112. Incubate at 4°C for 16 h under gentle agitation.
113. Decant primary antibody solution.
114. Add 10 mL of TBST to the container and incubate under gentle agitation for 10 min.
115. Decant the TBST into a waste container.
116. Repeat steps 114 and 115 two more times.
117. Dilute secondary antibody (anti-rabbit IgG:HRP) 1:2000 in TBST + 5% BSA, add to nitrocellulose membrane.
118. Incubate at room temperature for 1 h under gentle agitation.
119. Decant secondary antibody solution.
120. Add 10 mL of TBST to container and incubate under gentle agitation for 10 min.
121. Decant the TBST into a waste container.
122. Repeat steps 120 and 121 two more times.
123. Transfer the nitrocellulose membrane to a clean container.
124. Add 7 mL of Bio-Rad Clarity Western ECL substrate solution.
125. Incubate at room temperature for 5 min under gentle agitation.
126. Visualize using a Bio-Rad Chemidoc XRS+ system (or equivalent) (Figure 5).

EXPECTED OUTCOMES

Challenges associated with large scale production of bioactive growth factors have contributed to their high costs and role as a major cost driver in serum-free growth media. This issue is exacerbated within the nascent field of cellular agriculture which is projected to require large volumes of serum-free growth media supplemented with recombinant mitogenic growth factors. We have established a set of *E. coli* expression vectors that allowed for recombinant production of bioactive mitogenic growth factors in soluble form. The cloning and small-scale expression testing allow users to identify the most suitable approach for the expression of a specific growth factor family of interest, and then the generalized purification procedure allows for low-cost production at lab bench scale. In our study, we obtained yields in the mg/L range for all growth factor families tested including FGF2, IGF2, PDGF-BB and TGF-β1. All the growth factors we produced using this approach were biologically active and capable of promoting cellular proliferation in multiple cell systems. The Western

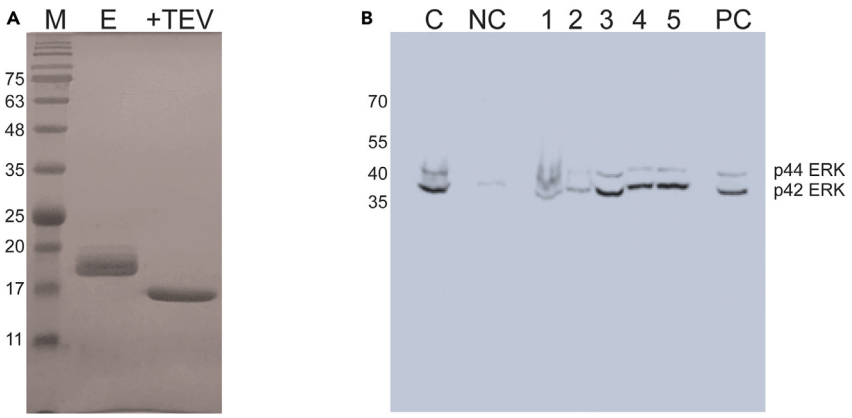

Figure 5. Representative results of purification and bioactivity screening

(A) SDS-PAGE of FGF2 purification. E – Elution; +TEV – 2nd Ni-NTA flowthrough collected after TEV protease cleavage. The shift in size of the band is due to the removal of the 6His and TEV recognition sequence.

(B) Western blot analysis of cell extracts treated with FGF2. C – commercial FGF2; NC – negative control – no FGF2; 1–5 – FGF2 orthologs; PC – positive control – 10% FBS. Panel B was adapted from[1].

blot assay to validate the bioactivity provides a quick and robust method for confirming activity of purified growth factors before their use in downstream applications. Our cost analysis suggested that purification of growth factors using this methodology will result in substantial cost reductions.

LIMITATIONS

This protocol relies on *E. coli* for eukaryotic growth factor expression. As a bacterial host, it lacks the machinery for most post-translational modifications present in eukaryotic organisms from which the growth factor sequences are derived. Consequently, if certain downstream applications of the end user necessitate these post-translational modifications, then this protocol may not be suitable for such applications.

A second limitation is related to the application of these growth factors in cellular agriculture, and specifically in cultured meat production. As a nascent technology, the regulatory framework(s) that will ultimately govern cultured meat has yet to be established in most jurisdictions. Consequently, the suitability of using recombinantly expressed growth factors in growth media for producing cultured meat intended for human consumption is undetermined at this stage. However, recombinant proteins produced in *E. coli* have a long track record of use in human health and food systems, and the potential for adventitious agents is substantially less than in bovine serum.

TROUBLESHOOTING

Problem 1

The growth factor protein is insoluble based on the results of small-scale expression testing.

Potential solution

Try alternative combinations of growth factor ortholog, expression vector, and host *E. coli* strain. For some of the families more recalcitrant to bacterial expression (e.g., IGF1, TGF-β1) we found that screening a variety of combinations was necessary to identify conditions for soluble expression. Expression conditions can also be modified, such as changing the IPTG concentration used for induction or changing the temperature during the induction phase.

Problem 2

TEV protease cleavage is incomplete.

Potential solution

Increase the ratio of TEV protease to growth factor protein (up to 60 μg TEV protease per mg of growth factor).

Problem 3

The growth factor is not present in the 2nd Ni-NTA Flowthrough.

Potential solution

Collect a wash fraction (30 mM imidazole) and a high imidazole fraction (250 mM imidazole) and analyze via SDS-PAGE to determine the location of the growth factor. Some orthologs can have an inherent ability to bind Ni-NTA at low imidazole concentrations and will remain bound to the matrix during flowthrough collection. In these instances, we find that the growth factors commonly elute in the wash buffer, with no impact on purity or protein functionality.

Problem 4

The Dsb[A/C] tag and the growth factor remain associated after TEV protease-mediated cleavage.

Potential solution

Dsb[A/C] promote proper disulfide bond formation and function as general chaperone-like proteins. Consequently, they often remain associated with growth factors after cleavage. When this occurs,

the growth factor will appear in the high imidazole wash of the 2nd Ni-NTA column (as the Dsb[A/C] fusion partner remains 6His-tagged). We found that the fusion proteins retain potent bioactivity when tested on multiple cell types (NIH-3T3 and bovine satellite cells). Dsb[A/C] fusion partners can also be separated from growth factors using a MonoQ anion exchange column.

Problem 5

The purified growth factor is prone to precipitation, decreasing the yield of bioactive product.

Potential solution

Add glycerol to the storage buffer and/or dilute the protein concentration.

Problem 6

The purified growth factor does not display bioactivity.

Potential solution

Ensure that the purified growth factor has been stored properly. If the problem persists, considering switching to an alternative ortholog.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alexei Savchenko, alexei.savchenko@ucalgary.ca.

Materials availability

Reagents generated as part of this study will be made available upon reasonable request.

Data and code availability

This study did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.S., A.S.; methodology and writing, C.S.; supervision, A.S.; funding acquisition, C.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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