Product Information

Description

Roke's Autoinduction/Autolysis *E. coli* strain is a proprietary bacterial strain that has been engineered for protein autoinduction resulting in high titers while also providing cell autolysis and nucleotide autohydrolysis capabilities to facilitate routine downstream workflows. Our strain leverages two-stage protein expression where cells grow until phosphate in the media becomes depleted. Once low phosphate conditions are reached, expression of the protein of interest is driven by the activation of low phosphate inducible promoters. In addition to the protein of interest, two chromosomally integrated accessory proteins are co-expressed: a lysozyme expressed in the cytosol (*λR* gene from bacteriophage lambda) and a nuclease expressed in the periplasm (*nucA* gene from *Serratia marcescens*). After cell harvesting, addition of a low amount of detergent (0.1% v/v) coupled with a freeze-thaw cycle results in >90% protein release and complete host oligonucleotides removal.

Strain Genotype

(F-, λ-, Δ(*araD-araB*)567, *lacZ4787(del)::rrnB-3*), *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR51, ΔackA-pta, ΔpoxB, ΔpflB, ΔldhA, ΔadhE, ΔicIR, ΔarcA, ΔompT::yibDp- λR-nucA-apmR*)

Advantages

-Reduces protein expression troubleshooting efforts
-Reduces hands-on time for expression and lysis
-Amenable for use in microtiter plates (384-well and 96-well plates), shake-flasks, or bioreactors
-Allows the processing of multiple biological samples without any lysis equipment
-Compatible with high-throughput assays (enzyme assays, capillary electrophoresis, etc.)

Protocol

IMPORTANT: For this protocol to work, it is required that your protein of interest is cloned under the control of a low phosphate inducible promoter. Routine cell dry weight values obtained in this protocol range from 3.4 to 6.8 grams cell dry weight (gCDW) (~10-20 OD_{600}) for microplates and shake-flasks. We provide a positive control expressing mCherry using a modified *yibD* gene promoter from a plasmid. This plasmid can be purified and used as a template for cloning your protein of interest and its plasmid map can be found at rokebio.com under Technical Resources. Optimal expression of mCherry from this plasmid is around 50% of total cell protein and can be confirmed using SDS-PAGE. For additional information on alternative low inducible phosphate promoters which result in high protein titers, please refer to Moreb, et. al., ACS Synth. Biol. 2020, 9, 6, 1483-1486.

Autoinduction in shake-flasks

-Start a 5 ml LB overnight culture using a frozen stock or a colony from an agar plate of Autoinduction/Autolysis *E. coli* strain expressing your protein of interest and incubate at 37 °C and 150 rpm (shaker orbit: 50 mm)
-Using the overnight culture, inoculate a 250 ml baffled vented shake-flask (e.g. VWR cat no. 89095-270) containing 20 ml of sterile AB-2 supplemented with appropriate antibiotics at 1% v/v (add 200 µl of the overnight culture).
-Incubate the flask(s) at 37 °C and 150 rpm (shaker orbit: 50 mm) for 24 hours.
-Measure and record the OD_{600} of the culture before harvesting by centrifugation at 4000 rcf for 20 minutes.

Autoinduction in microtiter plates

IMPORTANT: Microplate covers during incubation are needed to minimize evaporative losses (e.g. EnzyScreen Cat No. CR1596 for 96-well plates and EnzyScreen Cat No. CR1384 for 384-well plates)
-Fill a microtiter plate(s) with 15 µl or 100 µl of LB per well in a 384-well plate or 96-well plate respectively. For 384-well plates, supplement LB with 0.05% polypropylene glycol (MW: 2000 Da).
-For each protein of interest, inoculate one well with the Autoinduction/Autolysis *E. coli* strain expressing that protein and incubate overnight at 37 °C and 300 rpm (shaker orbit: 50 mm)
-Using the overnight cultures, inoculate 14.85 µl or 99 µl of sterile AB-2 per well, in a 384-well plate or 96-well plate respectively, at 1% v/v (add 0.15 µl or 1 µl of the overnight culture per well). For 384-well plates, supplement AB-2 with 0.05% polypropylene glycol (MW: 2000 Da).
-Incubate the plates at 37 °C and 300 rpm (shaker orbit: 50 mm) for 24 hours.
-Measure and record the OD600 of the culture before harvesting by centrifugation at 3000 rfc for 20 minutes.

Autoinduction in bioreactors

IMPORTANT: Since instrumented bioreactor setups and software vary widely, this section only provides general guidelines for performing fermentations in bioreactors. The volumes and control settings used below assume an 800 ml working volume vessel for a Multifors-2 bioreactor (Infors cat no. MUF2-M3-115) and may need to be adjusted accordingly.

Seed preparation

-Inoculate 50 ml of SM10 media in a 250 ml shake flask a with frozen stock or a colony from an agar plate of Autoinduction/Autolysis *E. coli* strain expressing your protein of interest.
-Incubate overnight at 37 °C and 150 rpm (shaker orbit: 50 mm).
-Measure the OD_{600}. To harvest the cells, an ideal OD_{600} range is between 5 and 10. Seeds made with cell cultures outside those values may result in extended lag times.
-Harvest the cells by centrifugation at 4000 rcf for 10 minutes, remove the media and resuspend the cells in fresh SM10 media adjusting the volume to a final OD_{600} of 10.
-Mix 6.5 of the resuspended cell culture with 1.5 ml of 50% sterile glycerol to make a bioreactor seed.

Bioreactor fermentation

-Fill the bioreactor with 740 ml of solution A of FGM10 or FGM25 (10 gCDW or 30 gCDW, respectively).
-Prepare and sterilize the bioreactor.
-After cooling, add 60 ml of sterile solution B of FGM10 or FGM25 supplemented with appropriate antibiotics.
-Adjust the pH of the media to 6.6-7.2, if needed.
-Inoculate the bioreactor with a fermenter seed.

Bioreactor oxygen and feeding control settings

Initial air flow rate is set at 0.3 vvm, initial agitation at 300 rpm and oxygen in the gas mixture at 0%. Dissolved oxygen is maintained at 25% by increasing airflow to a maximum of 1 vvm. If not sufficient, agitation is then increased to a maximum of 1200 rpm. If not sufficient, oxygen is then increased in the gas mixture to a maximum of 80%. Initial glucose feed rate is set to 0. For 10 gCDW fermentations, glucose is fed at 0.5 g/h once dissolved oxygen concentration drops from 100% to 25% and ramped up to 1 g/h, once increased agitation is triggered. For 30 gCDW, glucose is fed at 3.5 g/L-h when the agitation is increased above 1000 rpm. The glucose feed rate is then increased exponentially, doubling every 1.083 hours until a total of 40 g glucose has been added, at which point the set point is lowered to 1 g/L-h.

Autolysis

-Harvest the cells by centrifugation at 4000 rcf for 20 minutes.
-Resuspend the cells in autolysis buffer supplemented with protease inhibitors to a final OD_{600} of 200. We have not tested autolysis and autohydrolysis performance at a higher cell density.
-Freeze the cells for at least 1 hour in a -80 °C freezer.
-Incubate the cells at 37 °C for 1 hour.
-Clarify the lysate by centrifugation at 13000 rcf for one minute.
-To confirm nucleotide removal, one can examine a lysate sample using an agarose gel. There should not be any visible nucleotides.
-To confirm protein release, perform a Bradford or BCA assay. Protein concentrations should be in the order of micrograms per microliter.

Media and buffer formulations

AB-2

-Autoclave Solution A and B separately. After cooling, mix 90 ml of Solution A with 910 ml of Solution B.

Solution A (per liter)

Component	Final concentration
Glucose	500 g/L

Solution B (per liter)

Component	Final concentration
Bis-Tris	41.8 g/L
Ammonium sulfate anhydrous	5.4 g/L
Yeast Extract	6.2 g/L
Casamino acids	3.5 g/L
12 M HCl	3 ml/L

SM10

-After mixing the components, adjust pH to 6.8 with 10 M NaOH and filter sterilize

Per liter:

Component	Final concentration
10x Ammonium Citrate Salts, pH 7.5	100 ml/L
0.5 M Phosphate Buffer, pH 6.8	5 mM
Trace Metals 1	4 ml/L
Fe (II) Sulfate	0.16 mM
MgSO4	2.5 mM
CaSO4	0.0625 mM
Glucose	45 g/L
MOPS solution, pH 7.4	200 mM
Thiamine-HCl	0.01 g/L
Yeast Extract	1 g/L

FGM10

IMPORTANT: To facilitate conversions to different working volumes, final concentrations of each component in Solutions A and B reflect the final concentrations of such component in 1 liter of FGM10 (after mixing A and B together).

Solution A (per liter of FGM10). Sterilize by autoclaving.

Component	Final concentration
10x Ammonium Citrate Salts, pH 7.5	100 ml/L
Trace Metals 1	4 ml/L
Fe (II) Sulfate	0.16 mM
MgSO4	2.5 mM
CaSO4	0.0625 mM

Solution B (per liter of FGM10). Filter sterilize.

Component	Final concentration

0.5 M Phosphate Buffer, pH 6.8	10 ml/L
Glucose	25 g/L
Thiamine-HCl	0.01 g/L

FGM25

IMPORTANT: To facilitate conversions to different working volumes, final concentrations of each component in Solutions A and B reflect the final concentrations of such component in 1 liter of FGM25 (after mixing A and B together).

Solution A (per liter of FGM25). Sterilize by autoclaving.

Component	Final concentration
10x FGM25 Salts, pH 7.5	100 ml/L
Trace Metals 2	4 ml/L
Fe (II) Sulfate	0.36 mM

Solution B (per liter of FGM25). Filter sterilize.

Component	Final concentration
1.0 M Phosphate Buffer, pH 6.8	17.5 mM
Glucose	25 g/L
Thiamine-HCl	0.01 g/L

Autolysis buffer

Component	Final concentration
Tris-HCl, pH 8.0	20 mM
Triton X-100	0.1% (v/v%)

Media components

Below find the recommended stock solutions for each media component to prepare the formulations described above:

- 10x Ammonium Citrate Salts, pH 7.5: Mix 90 g ammonium sulfate anhydrous and 2.5 g citric acid. Adjust the volume to 1000 ml with water and pH to 7.5 with 10 M NaOH. Autoclave to sterilize.
- 10x FGM25 Salts, pH 7.5: Mix 90 g ammonium sulfate anhydrous, 2.5 g citric acid, 6.16 g magnesium sulfate heptahydrate, 0.11 g calcium sulfate dihydrate. Adjust the volume to 1000 ml with water and pH to 7.5 with 10 M NaOH. Autoclave to sterilize.
- 0.5 M Phosphate Buffer, pH 6.8: Mix 248.5 ml of 1 M potassium sulfate dibasic with 251.5 ml of 1 M potassium sulfate monobasic. Adjust to 1000 ml with water and filter sterilize.
- M Phosphate Buffer, pH 6.8: Mix 497 ml of 1 M potassium sulfate dibasic with 503 ml of 1 M potassium sulfate monobasic. Filter sterilize.
- Trace Metals 1: Mix 10 ml of 18 M sulfuric acid, 0.6 g cobalt sulfate heptahydrate, 0.5 copper sulfate pentahydrate, 2.4 g zinc sulfate heptahydrate, 0.2 g sodium molybdate dihydrate, 0.1 g boric acid and 0.3 g manganese sulfate monohydrate. Adjust to 1000 ml with water and filter sterilize.

- Trace Metals 2: Mix 20 ml of 18 M sulfuric acid, 2.4 g cobalt sulfate heptahydrate, 2 g copper sulfate pentahydrate, 2.4 g zinc sulfate heptahydrate, 0.8 g sodium molybdate dihydrate, 0.4 g boric acid, 1.2 g manganese sulfate monohydrate. Adjust to 1000 ml with water and filter sterilize.
- Fe (II) Sulfate: Mix 2.78 g Iron (II) sulfate heptahydrate and 2.5 ml 18 M sulfuric acid in water. Adjust to 100 ml with water and filter sterilize.
- 2 M Magnesium sulfate: Dissolve 246.47 g magnesium sulfate heptahydrate in water. Adjust to 500 ml with water and autoclave.
- 10 mM Calcium sulfate: Dissolve 1.72 g of calcium sulfate dihydrate in water. Adjust to 1000 ml with water and autoclave.
- 1 M MOPS: Mix 41.85 g of MOPS base in water. Adjust to 1000 ml with water and pH to 7.4 with 10 M NaOH. Filter sterilize.

Troubleshooting guide

Problem	Possible cause	Recommendation
Little or no expression of your protein of interest and mCherry control	Insufficient oxygen transfer	-Increase shaking rpm during incubation -Reduce the fill-volume at intervals of 10-25% of the total volume -Used baffled vented shake-flasks -Adjust the orbit of the shaker to 50 mm
	Incorrect media formulation	-Check the pH of the media is at 6.8-7.4 and adjust accordingly -Make fresh media (including fresh components)
	Strain is contaminated	-Streak your bacterial stock in a plate containing 100 µg/ml apramycin and appropriate plasmid antibiotic -Retransform your plasmid encoding your protein of interest in the Autoinduction/Autolysis strain
	Slow growth	-Increase cell culture incubation time to 36-48 hours
Little or no expression of your protein of interest, but optimal expression of mCherry control (around 50% of total protein)	Protein of interest is toxic	-Increase cell culture incubation time to 36-48 hours -Clone your protein of interest in a plasmid with a low copy origin -Clone a periplasmic signal peptide to your protein of interest to alleviate cell toxicity
	Suboptimal folding	-Repeat the protocol changing the incubation temperature and time to 30 °C and 48 hours, respectively. -Clone solubility tags to your protein of interest (SUMO, MBP, GST, etc)

		-Clone a periplasmic signal peptide to your protein of interest to allow for disulfide bonds
	Plasmid sequence is incorrect	-Using the mCherry control plasmid map and sequence, look for mutations in the promoter or RBS regions -Check and remove alternative RBS, deletions, or frameshifts
Expression of your protein of interest and mCherry control, but incomplete lysis or nucleotide removal	Low expression of accessory proteins	-Increase cell culture incubation time to 36-48 hours -After the freezing the cells during autolysis, increase the lysis incubation time at 1-hour intervals
	Cells are not frozen	-Ensure cells are frozen by tilting the tube -Freeze the cells overnight instead of 1 hour
	Incorrect lysis buffer	-Make fresh lysis buffer

For any questions or problems regarding the performance of this product, do not hesitate to contact us at support@rokebio.com. Additionally, if you have any suggestions about product performance or new applications, we encourage you to contact us at support@rokebio.com.

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