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Large scale EV-A71 2A protease mature and precursor expression setup and operation of Single Use Bubble Column Reactors: Litre-Scale Expression of Recombinant Proteins for Structural Biology and Drug Design (SBDD)

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We use this protocol and it's working

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Disclaimer

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Abstract

This protocol describes a single-use Bubble Column Reactor (suBCR) system for large-scale recombinant protein production in E. coli, addressing limitations of traditional shake-flask methods for structure-based drug discovery. The system enables parallel 1-liter batch cultivation using disposable bioreactor bags in a heated water bath with controlled aeration.

Two enterovirus 2A protease variants were successfully expressed: wild-type Human Coxsackievirus A16 2A protease (Addgene 228632) and an inactive Coxsackievirus A71 2A protease with C110A mutation (Addgene 228633).The C110A mutation preserves the VP1-2A junction while eliminating catalytic activity, providing active and inactive variants for comparative studies that are suitable for crystallography. Cultures are grown at 37°C for 4 hours until exponential phase, then expressed overnight at ambient temperature (25-27°C) using auto-induction Terrific Broth media. The system achieved high yields with culture volumes of 5-22.6 L producing wet cell weights of 163.2-225.6 g. Quality control via nickel-magnetic bead purification and SDS-PAGE confirmed successful overexpression of both constructs.

This scalable, cost-effective approach significantly advances protein production methodology for structural biology and drug discovery, offering increased throughput and reduced labor compared to conventional methods while providing sufficient protein quantities for crystallographic studies and antiviral drug development.

Attachments

PDF

PAGE24-00929 (1).pdf

1.1MB

Materials

Media (auto-induction):

Formedium AIMTB0310 **https://formedium.com/product/aim-terrific-broth-base-including-trace-elements/**

Ni-NTA Magnetic Beads (42179.02) from Serva (**https://www.serva.de/**)

Lysis reagent: FastBreak (V857C or V8571) from Promega (https://www.promega.co.uk/)

Protocol materials

Human Coxsackievirus A16 strain G10 2A protease **addgene Catalog #**228632

Enterovirus Coxsackievirus A71 inactive 2A protease with mutation C110A to preserve VP1-2A junction. **addgene Catalog #**228633

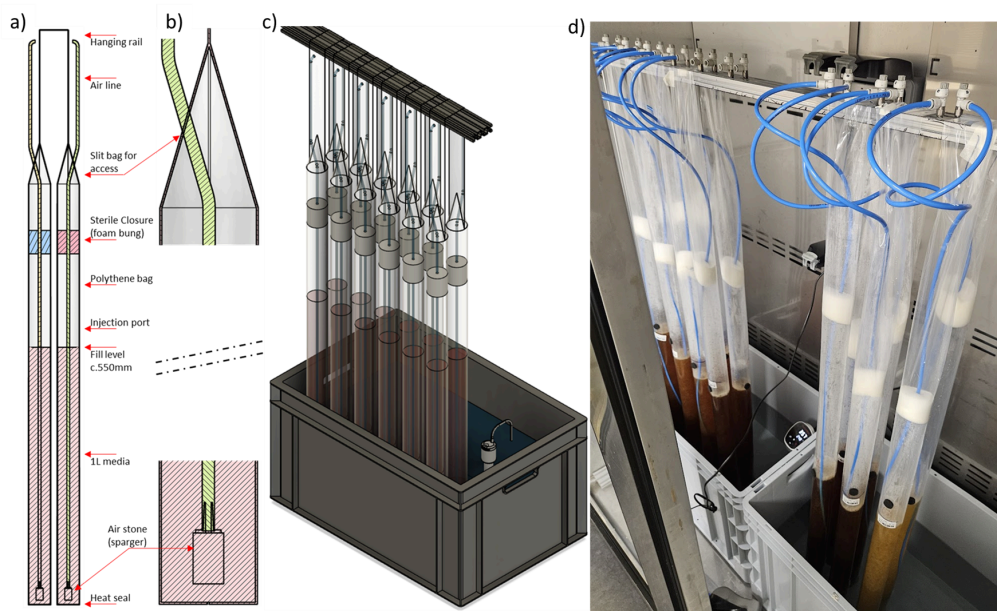
Before start

The reader should be familiar with the companion protocol: "The Manufacture and Setup of Single Use (SUB) Bubble Column Reactors (BCR): Litre-Scale Expression of Recombinant Proteins for Structural Biology and Drug Design (SBDD)."

Culture Main Bags

4h 32m

1



Bag setup: a) Guidance for Bag manufacture and assembly, b) Arrangement of the Bags over the hanging rail, c) A Bag Array in operation.

Bag set-up a) Bags removed from water bath for illustrative purposes, b) a typical bubble swarm and foam layer, c) close-up of the Bags in the water bath with heaters, d) a heat map showing water bath heating of the Bags;

with heaters, d) a heat map showing water bath heating of the Bags, left-to-right Bags acclimating.

2 **Prepare and autoclaved Terrific Broth Base including Trace elements (Formedium AIMTB310)**

2.1 To each litre of sterilised media:
Add 1 mL 10% Antifoam 204
Add 1 mL 1000x antibiotic stock (e.g. Kanamycin 50mg/ml)
Add 20 g glycerol

3 **Equilibrate the incubator bath:**

3.1 Start the immersion heater program 37 °C 06:00:00

3.2 Allow incubator to reach set temperature.

4 **Loading and equilibrating the BCR bags:**

4.1 Add 1 L prepared AIM TB media to each BCR bag.

4.2


The bag with airline inserted, and a funnel being used to demonstrate the filling process.

4.3 Transfer BCR bags to incubator.

4.4 Insert sparger stones into BCR bag, making sure they are at the bottom of the BCR.

4.5 Attach sparger stones to air control valves. Set air flow, 1 lpm.

4.6 Allow bags to equilibrate.

5 **Inoculate Media:**

5.1

Note

If cultures are innoculated by late morning biomass growth can be monitored in person. If choosing to manually induce protein expression by IPTG this can be done before the end of the work day.

Expression can run unattended overnight.

5.2 Add 10 mL starter culture, per L main culture.

6 **Biomass Growth Phase:**

6.1 Continue incubation 37 °C , 04:00:00 (approximately). 4h

Note

Incubation at 37 °C should continue until the culture has entered the exponential growth phase. Typically this takes 4-6 hours given the conditions described. Autoinduction media will trigger protein expression once all the glucose in the media has been consumed, this happens at OD600 of between 3-5 in most cases. The incubation temperature should be reduced before the autoinduction point to promote protein solubility.

Protein expression phase

7 **Protein Expression Phase we used the plasmid**

Human Coxsackievirus A16 strain G10 2A protease **addgene Catalog #**228632

and

Enterovirus Coxsackievirus A71 inactive 2A protease with mutation C110A to preserve VP1-2A junction. **addgene Catalog #**228633

7.1 Incubate (ambient) Overnight (approximately 16 h)

- [EndPoint] ΔOD600≈0

Note

Without external cooling cultures' metabolic activity will heat the culture to 25-27 °C. If working with proteins with temperature sensitive expression, an external recirculating chiller can be connected to the water bath.

7.2

Note

- A typical culture, having been innoculated at between 10-12 pm on day 1, can be harvested in the morning of day 2. This gives a total culture time of 20-24 hours.
- Culture times of up to 36 hours have been tried successfully, but this is likely to be cell/protein specific.
- Short expression experiments can be performed with a c.3 hour induction period following the addition of IPTG.
- Culture biomass can be measured by taking Optical Density readings at 600 nm in a spectrophotometer. The relationship between OD600 and biomass is non-linear at high concentrations. If using a fixed path-length or cuvette, dilute samples such that readings remain below 1 OD unit.
- If using autoinduction media and cell growth appears to have stalled below OD600=5, IPTG can be added to ensure induction has occurred.

7.3

Note

- In rich media biomass will continue to increase after induction.
- Once OD600 values plateau late log phase has occurred, and cell harvest must occur before OD600 begins to drop when death phase begins.

Harvest

8 **Harvest:**

8.1 Take final OD600 reading

8.2 Collect 1 mL culture samples for diagnostic testing

8.3 Transfer culture to 1 L centrifuge bottles

Note

The Bags are long and flexible, decanting by pouring culture out from the top of bags can lead to spillage.

Place Bags into a large jug or similar. With a sharp implement, make a small incision in the bag and allow it to drain into the jug.

There is a lot of hydrostatic pressure on the liquid at the bottom of the Bags when full. To avoid splashing make incisions towards the top of the liquid level at first. As the culture drains, larger incisions can be made at the bottom to drain all liquid and sediments.

8.4 Pellet the cell, 4000 rcf, 4°C, 00:30:00 30m

8.5 Drain spent media and sterilise with 1 tablet/L Virkon

8.6 Transfer cell pellet to cryo-safe sample bags. Mark with full and clear tracking information.

8.7 Record wet cell weight (g)

8.8 Flash freeze the cell pellet in liquid nitrogen.

8.9 Store cell pellet -80 °C

Overexpression quality control

9 **Overexpression QC:**

9.1

Note

Using Nickel Magnetic Beads perform a rapid over-expression assay. The protocol described here is abridged and achieves only incomplete cell lysis, but can be used to varify over-expression in 5-10 minutes, plus gel running time.

9.2 Transfer 1 ml of culture sample to a microcentrifuge tube go to step #8.2

9.3 Add 100 µL lysis reagent to the sample

> Note
>
> If biomass is low or protein expression levels are expected to be low, take a larger culture sample and pellet it first. This will increase the target protein yield but you may need to check sufficient lysis has occurred.

9.4 Incubate Room temperature 00:02:00 in a tube rotor

9.5 Pellet the cell debris at maximum speed in a microtube centrifuge
16000 rpm, 00:02:00

> Note
>
> There is no need to spin at 4 °C unless you plan to use the eluted protein for other purposes than QC SDS-PAGE.

9.6 Aspirate soluble fraction (**mixed proteins**) with pipette

9.7 Add 50 µL magnetic IMAC beads to the soluble fraction (**mixed proteins**)

9.8 Incubate Room temperature 00:05:00 in a tube rotor

9.9 Incubate Room temperature 00:01:00 in a magnetic separator stand

9.10 Aspirate soluble fraction (**unbound material**), reserve for SDS-PAGE analysis.

9.11 Wash the retained beads 2 mL binding buffer 00:01:00

9.12 Incubate Room temperature 00:01:00 in a magnetic separator stand

9.13 Aspirate soluble fraction (**unbound material**), reserve for SDS-PAGE analysis.

9.14 Add 50 µL elution buffer to the mag beads 2m

9.15 Incubate 00:02:00 Room temperature

9.16 Incubate 00:01:00 in a magnetic separator stand

9.17 Aspirate soluble fraction (**eluted protein**) with pipette

9.18 Measure the A280 using a spectrophotometer (e.g. NanoDrop)

9.19 Run an SDS-PAGE gel, to check for over-expression and quality control

Results

10 Tracking IDs and Yields:

	A	B	C	D	E	F	G	H	I	J
	ELN title	Target ID	Clone ID	Construct ID	Expression ID	Culture Total Volume (L)	OD600	Pellet Wet Cell Weight (g)	SDS-Page: Test purification Mag Prep	Expression Growth (Pass)

	A	B	C	D	E	F	G	H	I	J
									(pass)	
	Expression only A712A-c012	A71EV2A	A71EV2A-k005	A71EV2A-c012	A71EV2A-e078	12	22.6	225.6	Pass	Pass
	A71EV2A-c058	A71EV2A	A71EV2A-k030	A71EV2A-c058	A71EV2A-e067	5	18	163.2	Pass	Pass

11 Annotated SDS-PAGE gel of Mag Prep test purification