

Large Scale GFP Prep

Clark Lab

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Cell Growth

Start with fresh LB/Amp plate of BL21(DE3)pLysS cells containing the GFP plasmid (streak a fresh plate if necessary from glycerol freezer stock).

Use 50 ug/ml Amp in all media. 50 mg/ml Amp stock is in -20°C freezer in common room.

Grow overnight culture in 25 ml LB + 25 ul Amp, 37°C , shaking 170 rpm, in the dark.

Add 10 ml of overnight to 1 liter LB/Amp (1 ml of Amp) in 2L baffled flask

If you want 4 liters of culture, make 4 overnights and use 1 per 2L flask.

Check the OD 600 of each flask at 3 hrs, to decide whether to use the culture or not:

- If OD goes up too high, too fast, GFP does not express well.
- At 3hrs should be close to 0.2-0.5 AU. If much higher, abort that culture.
- Use the Baker Lab Agilent UV/Vis spectrophotometer to check the OD600

When OD 600 is between 0.5 and 0.7 AU:

- Induce expression of GFP by adding 1mM IPTG (0.238 g in 1ml filter sterilized for each 1L)
- Have frozen IPTG thawed before weighing, use sterile tubes and water, and filter sterilize before using.

Culture for 4 hours at 37°C , shaking in dark.

Cell Lysis

*** The following directions are for 1L culture... scale up as necessary! ***

Spin down cells (5K rpm, 10 min). Pour off supernatant (treat with lysol before rinsing down sink). Freeze cell pellet in -80°C freezer overnight (or longer).

Resuspend pellet in 20 ml of 50 mM phosphate, 300 mM NaCl pH8.0.

Add lysozyme to 1mg/ml (20 mg, lysozyme is in freezer)

Place resuspended cells with lysozyme in 50 ml glass beaker on ice. Cover with Parafilm.

Sonicate in FJC Lab using flat tip probe. Clean probe with water and kimwipe before and after use.

Control unit for sonicator is to bottom left of cabinet.

Sonicate with beaker in ice bucket.

Turn power on for 10 sec and off 50 sec.

Swirl beaker to mix cell suspension.

Repeat at least 4 times, or until solution is as thin as water. [It may take 15X or more.]

Transfer cell lysate to sterile SS34 centrifuge tube. Spin cells 15K, 15 min in JA-20 rotor. Save supernatant sample for gel analysis.

Resuspend pellet in 20 ml of 6 M GuHCl, 50 mM NaPO₄, 300 mM NaCl pH8.0.
You can shake overnight on shaker in cold room on low.

Remove small sample for gel analysis; dilute 1/25 and 1/100 in phosphate buffer to dilute down the GuHCl.

Make 1/25 and 1/100 dilutions of both pellet and supernatant for gel samples.
Run 10% SDS gel with dilute MWStd; silver stain.

Filter the resuspended pellet through at least a 0.45 um filter. [We are currently trying alternate filter systems.]

Chromatography

Prepare Ni-NTA Superflow (Qiagen) column on Akta

Ni-NTA = Nickel-nitrilotriacetic acid, 3cmX5.5cm

Set abs @ 280 on Akta screen.

Open PrimeView software on Compaq Laptop.

Make sure the computer and Akta are talking to one another.

Buffer recipes are listed after the method, below.

Column run at room temperature.

Buffers are filter sterilized and degassed. Long term storage in cold room, but used at room temperature.

Running Column: use manual method

Prepare column for use:

Wash with Lysis buffer >5 vol (150 ml, or to absorbance of 0)

Sometimes we do this overnight at 0.2 ml/min flow. Usual flow rate = 2 ml/min.

Use reservoir line A.

Load sample using the short line cannula in Injection Valve #8 at 2 ml/min.

Watch pressure, should not go up much over 0.1, can see peak on computer.

Wash sample with lysis buffer until peak comes down and absorbance is level over time.

Wash sample with wash buffer 5-10 column volumes; use line A (we run >240ml, usually 2-3 hrs at 2 ml/min). Keep washing until absorbance levels out.

Elute with gradient: 0-100% B over 90 minutes (elution buffer on reservoir line B) at 2ml/min.

Collect 8 ml fractions (can have fraction collector on before this, to collect wash).

GFP elutes at ~40% B in fairly sharp peak. If peak is not sharp, it is time to regenerate the column. Regeneration should be done after column is used 5 times. See instructions below, after Buffers.

Wash column in preparation of next run: overnight at 0.2 ml/min with Wash buffer. If not using again immediately, also wash column with sterile water (30ml), and then 30 minutes with 0.5 M NaOH @ 2ml/min, then store in 30% ethanol.

Prepare 10% SDS gel for analysis of fractions. Dilute samples 1/100 in phosphate buffer, then mix 20 ul dilute sample + 10 ul 3x loading buffer; load 20 ul on gel.

Can run samples of loading, washing as well as fractions.

Use dilute Molecular Weight Standard (marked Dil MWStd)

Silver stain.

Buffers

All buffers are filter sterilized and degassed, and stored long term at 4°C.

Use at room temperature. All reagents are from Fisher.

Lysis Buffer

50 mM NaPO₄ (6.9 g NaH₂PO₄+H₂O) (mw = 137.99)

6 M GuHCl (573.18 g) (mw = 95.53)

0.3 M NaCl (17.54 g) (mw = 58.44 mw)

10 mM imidazole (0.68 g) (mw = 68.08 mw)

dissolve in good (dd) water, on stir plate and pH to 8.0 with 50% NaOH qs to 1 liter and filter and degas for 20 minutes.

Wash Buffer

50 mM NaPO₄, 6 M GuHCl, 0.3 M NaCl, 20 mM imidazole, pH 8.0

Elution Buffer

50 mM NaPO₄, 6 M GuHCl, 0.3 M NaCl, 250 mM imidazole, pH 8.0

Usually need 1 liter each of Lysis and Wash and 200 ml of Elution per 1 Liter prep.

Regeneration of Ni-NTA Resin [From Qiagen Handbook]:

The reuse of resin should only be performed on identical recombinant proteins. The company recommends a maximum of 5 runs/column. If the Ni-NTA agarose changes from light blue to brownish-gray, the following regeneration procedure is recommended:

1. Wash with 2 volumes of regeneration buffer. [6 M GuHCl, 0.2 M acetic acid]
2. Wash with 5 column volumes H₂O.
3. Wash with 2 column volumes of 2% SDS.
4. Wash with 1 column volume of 25% EtOH.
5. Wash with 1 column volume of 50% EtOH.
6. Wash with 1 column volume of 75% EtOH.
7. Wash with 5 column volumes of 100% EtOH.
8. Wash with 1 column volume of 75% EtOH.
9. Wash with 1 column volume of 50% EtOH.
10. Wash with 1 column volume of 25% EtOH.
11. Wash with 1 column volume H₂O.
12. Wash with 5 column volumes of 100 mM EDTA, pH 8.0.
13. Wash with H₂O.
14. Recharge column with 2 volumes of 100 mM NiSO₄.
15. Wash with 2 column volumes of H₂O.
16. Wash with 2 column volumes of Regeneration buffer.
17. Equilibrate with 2 volumes of starting buffer.