

Studier Method for Induction

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Author: based on Bill Studier's Protocol

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| Materials/Reagents/Equipment | |
|---|---|
| Reagents | Vector: Has to have a T7 lac Promoter, i.e. |
| For all media: | pET vectors |
| 1 M MgSO ₄ | Background strain: BL21(DE3) or B834(DE3)—both work in ZYM-5052 or PASM-5052. |
| 50xM | |
| Antibiotics | |
| (Particular media) | |
| 40% glucose (MDG) | 40 g/100 ml water, autoclave |
| methionine (MDG, PASM-5052) | 25mg/ml, filter sterilize |
| 17amino acids [aa] (MDG, PASM-5052) | Mix and filter sterilize |
| 50x5052 (PASM-5052, ZYM-5052) | |
| ZY (ZYM-5052) | |
| Se-Met (PASM-5052) | 25 mg/ml |
| vitamin B12 (PASM-5052) | |
| 1000X trace metals mix (PA-0.5g, PASM-5052) | |

Procedure:

MDAG plates: This media will inhibit leaky expression of target gene.

To make 500 ml (approx. 20 ml per plate yields 25 plates)

7.5 g agar

450 ml H₂O

Autoclave 15 min., mix well, let cool approx. 10 min on bench

Add the following sterile solutions:

1 ml 1M MgSO₄ @ 2mM

100 µl 1000x metals @ 0.2 x

6.25 ml 40% glucose @ 0.5%

25 ml 5% Aspartate @ 0.25%

10 ml 50xM @ 1X M

10 ml 17aa (10 mg/ml ea.) (no C, Y, M) @ 200 µg/ml of each

4 ml Met (25 mg/ml) @ 200ug/ml

Selective antibiotic(s), if desired.

Pour plates, incubate overnight at 37°C to test for contamination, store at 4C.

Starter in non-inducing media: MDG

Grow strains to saturation in MDG at 37°C (overnight at A_{600} ~4-7, pH ~6.5)
Store at 4°C until ready to use. This is stable at 4°C for 3-4 weeks.

For 200 ml of MDG

IMPORTANT: When using B834 auxotrophs, be sure to add 200 ug/ml of Met.

Autoclave 183 ml water

Add these sterile ingredients in the following order:

1. 400 μ l 1 M $MgSO_4$ and 40 μ l of 1000 X trace metals mix.
2. Add the following:
2.5 ml of 40% glucose @ 0.5%
10 ml of 5% aspartate @ 0.25%
4 ml of 50xM @ 1X
Antibiotics as needed.

For all media:

- Aeration in rotary incubator at 210 rpm for 500 ml cultures in 1.8-2.8 L Fernbach flasks. (1.8 L recommended by Studier)
- Dilute **1000 fold** from MDG into the auto-inducing media (ZYM-5052 or PASM-5052)

Important: Induction occurs when cells start using lactose as a C source and when the oxygen level becomes limiting.

For ZYM-5052 Media (complex auto inducing media; metals are optional at 0.2 X to 0.5 X):

Day 1 Inoculate at a 1:1000 dilution into ZYM-5052 media at 9am. Shake at 210 rpm for 5-6 hrs at 37°C until turbid. Bring temperature to 20°C, shake overnight. Induction takes place when cells are close to saturation.

Day 2 9am: Check OD_{600} . Continue to grow.
11am: Check OD_{600} . If OD has not changed, you could harvest then or wait a few more hours before harvesting.
If OD has decreased, harvest right away.
If OD has increased, continue to let cells grow until OD plateaus.
This all depends on each individual clone.

For PASM-5052 Media:

Day 1 Inoculate at a 1:1000 dilution into PASM-5052 media at 9am. Shake at 210 rpm for 5-6 hrs at 37°C until turbid. Bring temperature to 20°C, shake overnight.

Day 2 9am: Check OD_{600} . Continue to grow if OD is below 8-10. At low temperatures, BS says that it may take 5 days to reach desired OD. At 37°C, it will take less time (afternoon of day 2).

For small scale mini-expression:

We have inoculated directly from a colony into a 24 well Grenier Bio-One plate (1ml of ZYM-5052), allowed the cells to grow at 37°C for 7 hrs and obtained fairly good expression, but probably not optimum. You can also use 96 well plates (2 ml wells) with 0.25 ml media.

ZYM-5052 (complex auto-inducing media, 50 mM Phosphate) Inoculum: cells grown in MDG diluted 1:1000 fold

-5hours incubation at 37°C, 20 hrs at 20°C

For 500 ml of ZYM-5052:

Autoclave 479 ml ZY media

Add these sterile solutions in the following order:

1 ml 1 M MgSO₄ @ 2 mM and 100 ul of 1000X trace metals @0.2 Xmix.

Then add the following:

10 ml 50x5052

10 ml 50xM

Antibiotics as needed

PASM-5052 (medium for Se-Met labeling, 100 mM Phosphate) Inoculum: cells grown in MDG diluted 1:1000 fold into PASM-5052 media

-5 hours incubation at 37°C, Incubation at 20°C may take upwards of 120 hrs to obtain an OD₆₀₀ between 8-10.

For 500ml:

Autoclave 450 ml sterile water

Add these sterile ingredients in the following order:

1 ml 1 M MgSO₄ @ 2 mM and 100 µl of 1000X @0.2 X trace metals mix

10 ml 50X 5052 @ 0.5% glycerol; 0.05% glucose; 0.2% lactose

25 ml 20xP @ 50 mM Na₂HPO₄, 50 mM KH₂PO₄, 25 mM(NH₄)₂SO₄

0.5 ml 100 µM vitamin B12 @ 100 nM

200 µl methionine (25 mg/ml) @ 10 µg/ml

2.5 ml Se-Met (25 mg/ml) @ 125 µg/ml

10 ml 17aa @ 200ug/ml (no C, Y, M)

Antibiotics as needed

Recipes:

Stock Solutions

Use deionized distilled water for all solutions
Autoclave solutions for 15 min unless specified otherwise

ZY

10 g N-Z-amine AS (or any tryptic digest of casein, e.g. tryptone)

5 g yeast extract
1000 ml water

20xP: (1 X P = 100 mM PO₄, 25 mM (NH₄)₂SO₄)

To make 100 ml:

90 ml water
6.6 g (NH₄)₂SO₄
13.6 g KH₂PO₄
14.2 g Na₂HPO₄

To make 1 liter:

900 ml water
66 g (NH₄)₂SO₄ = 0.5 M
136 g KH₂PO₄ = 1 M
142 g Na₂HPO₄ = 1 M

add in sequence in beaker, stir until all dissolved
pH of 20-fold dilution in water should be ~6.75

50xM: (1xM = 50 mM PO₄, 50 mM NH₄Cl, 5 mM Na₂SO₄)

To make 100 ml:

| | MW | 50x | 1x |
|--|-------|--------|-------|
| 80 ml H ₂ O | | | |
| 17.75 g Na ₂ HPO ₄ | 142.0 | 1.25 M | 25 mM |
| 17.0 g KH ₂ PO ₄ | 136.1 | 1.25 M | 25 mM |
| 13.4 g NH ₄ Cl | 53.49 | 2.5 M | 50 mM |
| 3.55 g Na ₂ SO ₄ | 142 | 0.25 M | 5 mM |

pH of 50 fold dilution should be ~ 6.7; occasionally has showered crystals which re-dissolve in the microwave.

50x5052: (1 X 5052 = 0.5 % glycerol, 0.05% glucose, 0.2% alpha-lactose)

To make 100 ml:

25 g glycerol (weigh in beaker)
73 ml water
2.5 g glucose
10 g α-lactose

To make 1 liter:

250 g glycerol (weigh in beaker)
730 ml water
25 g glucose
100 g α-lactose

add in sequence in beaker, stir until all dissolved
lactose is slow to dissolve-- may take two hours or more at room temperature, can speed up by heating in microwave oven

1 M MgSO₄

24.65 g MgSO₄·7H₂O
water to make 100 ml

40% glucose (w/v)

| | |
|------------------------|------------------------|
| <u>To make 100 ml:</u> | <u>To make 300 ml:</u> |
| 74 ml water | 222 ml water |
| 40 g glucose | 120 g glucose |

add glucose to stirring water in beaker
stir until all dissolved -- may take 45 minutes or more at room temperature can speed up by heating in microwave oven

80% glycerol (v/v) (= 100% w/v)

80 g glycerol (weigh in beaker)
20 ml water Final volume: 100 ml

20% alpha-lactose (w/v)

| | |
|------------------------|------------------------|
| <u>To make 100 ml:</u> | <u>To make 600 ml:</u> |
| 87.5 ml water | 525 ml water |
| 20 g α -lactose | 120g α -lactose |

add lactose to stirring water in beaker
stir until all dissolved -- may take 2 hours or more at room temperature can speed up by heating in microwave oven

5 % aspartate (sodium salt)

100 ml
97 ml water
5 g aspartic acid (sodium salt) MW: 133, 376 mM
~ 1.6 g NaOH, pH: neutral

Amino Acids

Methionine

25 mg/ml, autoclave 15 min MW: 149.2; conc. 168 mM

17aa (CYM) (10 mg/ml each) (contains no Cys, Tyr, Met)

Store in refrigerator

To 90 ml water in a beaker on a magnetic stirrer, add 1 g each of the following 17 amino acids in the order shown:

| | | |
|---|---------|---|
| 1 | Na Glu | E |
| 2 | Asp | D |
| 3 | Lys-HCl | K |
| 4 | Arg-HCl | R |
| 5 | His-HCl | H |
| 6 | Ala | A |
| 7 | Pro | P |
| 8 | Gly | G |

| | | |
|----|-----|---|
| 9 | Thr | T |
| 10 | Ser | S |
| 11 | Gln | Q |
| 12 | Asn | N |
| 13 | Val | V |
| 14 | Leu | L |
| 15 | Ile | I |
| 16 | Phe | F |
| 17 | Trp | W |

Asp is slow to dissolve, may need other amino acids for pH balance?

Continue to add the other amino acids, which will dissolve completely

Val, Leu, Ile float on the surface

increase the stirring rate to submerge and dissolve

Stir until everything dissolves to a clear solution before adding

Trp

Trp we have is slightly brown flakes

turns solution light brown upon dissolving

some material remained undissolved?

Filter sterilize

most brown material remained on filter

resulting solution was almost colorless (very light brown)

Vitamins

100 μ M vitamin B12

13.55 mg vitamin B12 MW 1355

100 ml autoclaved water

Filter sterilize, store in refrigerator.

Studier Media stock solutions:

Autoclave all stock solutions unless otherwise noted:

20 x P

900 ml water

| Chemical (for 1L) |
|-------------------|
|-------------------|

| |
|--|
| (NH ₄) ₂ SO ₄ 66 g |
|--|

| |
|---------------------------------------|
| KH ₂ PO ₄ 136 g |
|---------------------------------------|

| |
|--|
| Na ₂ HPO ₄ 142 g |
|--|

pH: a 20 fold dilution gives pH of ~6.75

Add in order, water first

50xM

Studier media
8/10/05 R. K.

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800 ml water

| Chemical (for 1L) |
|--|
| NH ₄ Cl 134 g |
| Na ₂ SO ₄ 35.5 g |
| KH ₂ PO ₄ 170 g |
| Na ₂ HPO ₄ 177.5 g |

pH: a 50 fold dilution should be approx. 6.7.

Occasionally has showered crystals, which re-dissolve in microwave.

50x 5052

| Chemical |
|-------------------------------|
| 250 g glycerol @ 25% |
| 730 ml water |
| 25 g glucose @ 2.5% |
| 100 g α -lactose @ 10% |

1000X Trace Metals Mix (100 ml; slight precipitate forms with time)

Add to 36 ml sterile water

All stock solutions of metals except 0.1 M FeCl₃-6H₂O are autoclaved. Store at room temp.

| Chemical | Volume | MW | Final [] | |
|---|--------|---------|-----------|-----------|
| 0.1 M FeCl ₃ -6H ₂ O (dissolved in 0.1 M HCl, 100 fold dilution of conc. HCl) | 50 ml | 270.30 | 50 uM Fe | |
| 1 M CaCl ₂ | 2 ml | 93.0 | 20 uM Ca | 1.1g/10ml |
| 1 M MnCl ₃ -4H ₂ O | 1 ml | 197.91 | 10 uM Mn | 1.97g |
| 1 M ZnSO ₄ -7H ₂ O | 1 ml | 287.56 | 10 uM Zn | 2.87g |
| 0.2 M CoCl ₂ -6H ₂ O | 1 ml | 237.95 | 2 uM Co | 0.476g |
| 0.1 M CuCl ₂ -2H ₂ O | 2 ml | 170.486 | 2 uM Cu | 0.17g |
| 0.2 M NiCl ₂ -6H ₂ O | 1 ml | 237.72 | 2 uM Ni | 0.475g |
| 0.1 M Na ₂ MoO ₄ -5H ₂ O | 2 ml | 241.98 | 2 uM Mo | 0.24g |
| 0.1 M Na ₂ SeO ₃ -5H ₂ O | 2 ml | 263.03 | 2 uM Se | 0.263g |
| 0.1 M H ₃ BO ₃ | 2 ml | 61.83 | 2 uM B | 0.06g |

Filter Sterilize.

Facts from B. Studier (6/2/03)

For PASM-5052 media: BL21(DE3) is as good as B834 (DE3).

PASM-5052 gives yields comparable to ZYM-5052.

After induction, it is usually OK if the cells keep growing longer. At times, he has seen that induction does take place after prolonged incubation.

In PASM-5052, at 20C, it may take 5 days to achieve induction.

Recommended that you take samples and run a gel to check for induction before harvesting. Try letting cells grow another 24 hrs.

He also sees complete Se-met incorporation with PASM-5052.

We did not find the baffled flask to be better than the regular flasks. We use 2.8 L Fernbach flasks.

Antibiotics:

Kanamycin (25 mg/ml in water, filter sterilize; final: 25 µg/ml)

Chloramphenicol (25mg/ml in 95% ETOH; final: 25 µg/ml)

Ampicillin or carbenicillin (100 mg/ml in water, filter sterilize; final: 100 µg/ml)

Spectinomycin (30 mg/ml in water, filter sterilize, final: 30 µg/ml)

Tetracycline (12.5 mg/ml in 50% ETOH; filter sterilize, final: 12.5 µg/ml)

ALERT:

BL21(DE3), no plasmid, in presence of PO₄ rich media, high amino acids, are resistant to 25 µg/ml Kan (ZYP-5052 media)

In ZB or ZYB, BL21(DE3) gets killed by Kan.

In ZYM 5052, use 100ug/ml Kan.

To measure pH of media, dilute 1:10 in water. Check pH pre and post growth of cells.