Studier Method for Induction

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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:

<u>MDAG plates:</u> 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 H20
Autoclave 15 min., mix well, let cool approx. 10 min on bench

Add the following sterile solutions:
- **1 ml 1M MgS04 @ 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.

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

For 200 ml of MDG

Autoclave 183 ml water
Add these sterile ingredients in the following order:
1. 400 µl 1 M $MgSO_4$ and 40 µl 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 $MgSO_4$ @ 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 OD_{600} between 8-10.
For 500ml:
Autoclave 450 ml sterile water
Add these sterile ingredients in the following order:
1 ml 1 M $MgSO_4$ @ 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)2S04
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 PO_4, 25 mM $(NH4)_2SO_4$)

To make 100 ml:	To make 1 liter:
90 ml water	900 ml water
6.6 g $(NH_4)_2SO_4$	66 g $(NH_4)_2SO_4$= 0.5 M
13.6 g KH_2PO_4	136 g KH_2PO_4 = 1 M
14.2 g Na_2HPO_4	142 g Na_2HPO_4 = 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 PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4)

To make 100 ml:

	MW	50x	1x
80 ml H_20			
17.75 g Na_2HPO_4	142.0	1.25 M	25 mM
17.0 g KH_2PO_4	136.1	1.25 M	25 mM
13.4 g NH_4CL	53.49	2.5 M	50 mM
3.55 g Na_2SO_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:	To make 1 liter:
25 g glycerol (weigh in beaker)	250 g glycerol (weigh in beaker)
73 ml water	730 ml water
2.5 g glucose	25 g glucose
10 g a-lactose	100 g a-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 $MgSO_4$

24.65 g $MgSO_4$-$7H_2O$
water to make 100 ml

40% glucose (w/v)

To make 100 ml:	To make 300 ml:
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)

To make 100 ml:	To make 600 ml:
87.5 ml water	525 ml water
20 g α-lactose	120g a-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

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 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)
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Vitamins

100 µM 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)
$(NH_4)_2SO_4$ 66 g
KH_2PO_4 136 g
Na_2HPO_4 142 g

pH: a 20 fold dilution gives pH of ~6.75
Add in order, water first

50xM

800 ml water

Chemical (for 1L)
NH_4CL 134 g
Na_2SO_4 35.5 g
KH_2PO_4 170 g
Na_2HPO_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 $FeCl_3$-$6H_3O$ are autoclaved. Store at room temp.

Chemical	**Volume**	**MW**	**Final [ ]**	
0.1 M $FeCl_3$-$6H_3O$ (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 $MnCl_3$-$4H_3O$	1 ml	197.91	10 uM Mn	1.97g
1 M $ZnSO_4$-$7H_3O$	1 ml	287.56	10 uM Zn	2.87g
0.2 M $CoCl_2$-$6H_3O$	1 ml	237.95	2 uM Co	0.476g
0.1 M $CuCl_2$-$2H_3O$	2 ml	170.486	2 uM Cu	0.17g
0.2 M $NiCl_2$-$6H_3O$	1 ml	237.72	2 uM Ni	0.475g
0.1 M Na_2MoO_4-$5H_3O$	2 ml	241.98	2 uM Mo	0.24g
0.1 M Na_2SeO_3-$5H_3O$	2 ml	263.03	2 uM Se	0.263g
0.1 M H_3BO_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 PO_4 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.