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Protein Production by Auto-Induction in High-Density Shaking Cultures

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Abstract. Inducible expression systems in which T7 RNA polymerase transcribes coding sequences cloned under control of a T7*lac* promoter efficiently produce a wide variety of proteins in *Escherichia coli*. Investigation of factors that affect stability, growth and induction of T7 expression strains in shaking vessels led to the recognition that sporadic, unintended induction of expression in complex media, previously reported by others, is almost certainly caused by small amounts of lactose. Glucose prevents induction by lactose by well-studied mechanisms. Amino acids also inhibit induction by lactose during log-phase growth, and high rates of aeration inhibit induction at low lactose concentrations. These observations, and metabolic balancing of pH, allowed development of reliable non-inducing and auto-inducing media in which batch cultures grow to high densities. Expression strains grown to saturation in non-inducing media retain plasmids and remain fully viable for weeks in the refrigerator, making it easy to prepare many freezer stocks in parallel and use working stocks for an extended period. Auto-induction allows efficient screening of many clones in parallel for expression and solubility, as cultures have only to be inoculated and grown to saturation, and yields of target protein are typically several-fold higher than obtained by conventional IPTG induction. Auto-inducing media have been developed for labeling proteins with selenomethionine, ^{15}N or ^{13}C, and for production of target proteins by arabinose induction of T7 RNA polymerase from the pBAD promoter in BL21-AI. Selenomethionine labeling was equally efficient in the commonly used methionine auxotroph B834(DE3) (found to be *metE*) or the prototroph BL21(DE3).

Growth media have been developed to control the expression of target genes in *E. coli* strains such as BL21(DE3), in which T7 RNA polymerase expressed from the inducible *lacUV5* promoter in the chromosome directs the expression of target protein from the T7*lac* promoter in multi-copy pET expression vectors (Studier *et al.*, Methods in Enzymology 185: 60-89 (1990)). These media should also be applicable to other strains where expression is controlled by promoters inducible by lactose or IPTG. Media are also described for auto-induction of promoters inducible by arabinose.

Non-inducing media. Fully defined non-inducing media give reliable growth without detectable induction of target protein all the way to saturation. They also support growth to higher cell densities than typical of complex media such as LB (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) and produce cultures that remain highly viable for weeks in the refrigerator. Even strains expressing target proteins highly toxic to the host remained stable in these non-inducing media, grew sub-cultures with little lag and were fully competent to express target protein. In contrast, unintended spontaneous induction can occur in typical complex media (Grossman *et al.*, Gene 209: 95-103 (1998)), which can stress or kill productive cells and favor poorly expressing derivatives. The common practice of using cultures grown to saturation in LB or other complex media to produce seed cultures for production of target protein may be responsible for many cases of poor expression in IPTG-inducible expression systems. Although addition of glucose can prevent such induction, finding a glucose concentration that reliably prevents induction in complex media without cultures becoming very acidic at saturation proved difficult or impossible.

Unintended induction is almost certainly due to small amounts of lactose present in enzymatic digests of casein, such as tryptone or N-Z-amine, in commonly used media such as LB. Casein comes from milk, which contains lactose, and different lots of purified casein used to make growth media may well contain different levels of residual lactose. The high concentrations of amino acids in such media suppress induction by lactose during log-phase growth, but less than 0.001% lactose can cause induction upon approach to saturation in cultures grown at moderate levels of aeration. Such inducing activity appears to be relatively common in commercial growth media made from enzymatic digests of casein. Therefore, isolation of expression strains, growth of freezer stocks for long-term storage, and growth of seed stocks for protein production should all be done in reliable non-inducing media such as MDG or MDAG.

Auto-inducing media. Media such as ZYM-5052 and MDA-5052 have been formulated to grow IPTG-inducible expression strains, initially without induction, and then to induce production of target protein automatically, usually near saturation at high cell density. A limited concentration of glucose is metabolized preferentially during growth, which prevents uptake of lactose until the glucose is depleted, usually in mid to late log phase. As the glucose is depleted, lactose can be taken up and converted by β-galactosidase to the inducer allolactose. Allolactose causes release of *lac* repressor from its specific binding sites in the DNA and thereby induces expression of T7 RNA polymerase from the *lacUV5* promoter and unblocks T7*lac* promoters, allowing expression of target proteins by T7 RNA polymerase. Depletable glucose can also allow auto-induction of arabinose-inducible promoters by arabinose in the medium, and the method could also be applied to promoters regulated by other metabolites subject to catabolite repression or inducer exclusion. Even strains that produce target proteins highly toxic to the host cell can grow normally and express their protein by auto-induction. Strains defective in *lacZ* (β-galactosidase) or *lacY* (lactose permease) are not likely to be suitable for auto-induction by lactose because they will be unable to import lactose or convert it to allolactose.

Lactose itself is not a particularly good carbon and energy source to support continued target protein production after auto-induction, because production of target protein competes so successfully for resources that proteins of the lactose operon may not accumulate to levels that provide efficient use of lactose. Glycerol is a good carbon and energy source that does not interfere with induction and allows growth to much higher culture densities. As with glucose, metabolism of glycerol generates acid, but very high densities of auto-induced cultures can be achieved with sufficient aeration and maintenance of a pH near neutral.

Growth conditions. Reasonably good aeration is important for maintaining pH near neutral and obtaining growth to high culture densities. We typically grow cultures in an incubator shaker at 20°C or 37°C and 300-350 rpm, using vessels and volumes of culture that give approximately equivalent levels of aeration. For auto-induction of many cultures in parallel to test expression and solubility, we grow 0.5 ml of culture in 13x100 mm glass culture tubes. Usually only a few microliters of such cultures is sufficient for all needed analyses by gel electrophoresis. Up to 2.5 ml of culture in non-inducing media is grown in 18x150 mm glass culture tubes to make freezer stocks, plasmid preps or seed stocks for moderate-scale auto-induction, although 1.5 ml is probably preferable. Seed stocks for larger-scale auto-induction can be grown in Erlenmeyer flasks, the culture occupying approximately 5-10 % of the flask volume. Moderate-scale auto-induction can use 400-500 ml of culture in 1.8-liter baffled Fernbach flasks (Bellco).

Trace metals. Growth to high density in fully defined media requires the addition of trace metals. A concentration of 0.2x trace metals (recipe for 1000x stock solution on page 19) is sufficient but, if a mixture of trace metals is not available, 100 μM $FeCl_3$ will increase saturation density about as well. Growth in media containing ZY has not been limited by lack of trace metals but, given the variability of complex media components, it seems prudent to add 0.2x trace metals, if available. For target proteins of unknown metal content, 1x trace metals provides nine different metals in amounts sufficient to saturate substantial production of target protein, and 5x can be tolerated with little effect on growth. Individual metals can be supplied for production of target proteins known to bind specific metals. Trace metal mix at 1x concentration or $FeCl_3$ at concentrations higher than about 10 μM tend to precipitate slowly in growth media. Such precipitation does not seem to inhibit growth but might decrease availability to metal-binding target proteins. The presence of 1 mM citrate seems to reduce or prevent such precipitation.

Additives. Fully defined media must be appropriately supplemented for growth of strains with nutritional requirements: at least 150 μg/ml of methionine is needed to support growth of B834(DE3) to saturation in MDG , and 1 μM thiamine (vitamin B1) is 10-fold more than needed to support high-density growth of XL1Blue-MR, an F^- *recA1* strain we use as an initial recipient for expression plasmids. I also found that growth of cultures in most batches of complex media such as LB, 2xYT or terrific broth is limited by lack of magnesium. Simply adding 2 mM $MgSO_4$ typically increased saturation densities by 50% to 5-fold.

Kanamycin resistance. Although BL21(DE3) is unable to grow in LB containing as little as 10 μg of kanamycin per ml, it grows well even at ten-fold higher levels of kanamycin in the comparably rich auto-inducing medium ZYP-5052, which contains 100 mM phosphate. However, kanamycin at 100 μg/ml does prevent growth in media reformulated to contain only 50 mM phosphate (or less), such as the auto-inducing medium ZYM-5052 we use currently.

Freezer stocks for long-term storage of expression strains are made by adding 0.1 ml of 100% w/v (80% v/v) glycerol to 1 ml of culture that was grown to saturation in a non-inducing medium such as MDG or MDAG-135, mixing well and placing in a -70°C freezer. Reasonably well aerated cultures in MDG or MDAG at 37°C typically saturate at an OD_{600} around 7-10. Working stocks are grown from freezer stocks by scraping up a small amount of frozen culture with a sterile plastic pipettor tip and inoculating non-inducing medium. The stability, viability and reliability of protein expression from cultures grown in non-inducing media makes it possible to work with many strains in parallel. Re-transformation or streaking out cultures to obtain a "fresh" single colony each time a protein is to be produced, an unfortunate and tedious practice in some labs, is not necessary for reliable expression of target protein.

Auto-induction works well over the entire range of temperatures suitable for growth. Auto-inducing cultures are typically inoculated with one-thousandth volume of an MDG or MDAG seed culture and grown to saturation overnight at 37°C. In general, increasing the rate of aeration increases the density at which the culture auto-induces and saturates, and also increases the minimum concentration of lactose needed for good auto-induction. Routine auto-inducing media contain 0.2% lactose, a concentration chosen to be well in excess of that needed for good auto-induction over the range of conditions we use.

Cultures grown at 20°C usually auto-induce and saturate at higher culture densities at than at 37°C (probably due to the higher solubility of oxygen at lower temperature). Higher saturation densities combined with slower growth at low temperature means that cultures may become quite dense after overnight incubation but may not yet be induced, so care must be taken not to collect low-temperature cultures before they have saturated. The time needed for auto-induction at low temperatures can be shortened by incubating a few hours at 37°C, until cultures become lightly turbid (probably OD_{600} less than 1), and then transferring to the lower temperature.

Incubation for several hours at saturation after auto-induction usually has little effect on accumulation or solubility of target protein. Auto-induced cultures typically saturate at an OD_{600} around 7-10 at 37°C under the conditions we use but can reach 20-30 in favorable cases. Culture densities greater than OD_{600} ~50 have been attained by using higher concentrations of glycerol, metabolic balancing of pH with aspartate or succinate, and higher levels of aeration. Auto-inducing media should be capable of producing even higher densities in batch culture in fermenters, where high levels of aeration can be maintained for large culture volumes. When the target protein is sufficiently toxic to the host, expression of even small amounts as auto-induction begins may prevent much further increase in density, and such cultures may not achieve densities much higher than OD_{600} of 1-2. Because the OD_{600} is due to light scattering rather than absorption, an accurate reading requires dilution of the culture (in water) to a concentration that gives a reading between about 0.030 and 0.200, typically a 100-fold dilution.

Parallel growth of many non-induced or auto-induced cultures is feasible because cultures are simply inoculated and grown to saturation. This is a great convenience and simplifies manual or automated induction and analysis of multiple clones compared to conventional IPTG induction, which requires monitoring growth of each culture and adding inducer at the proper stage of growth. Others have been successful using these recipes in the multi-well plates commonly used in automated systems, but it was important to keep volumes low enough that reasonably good mixing and aeration was obtained.

Stock solutions were designed for convenience and flexibility in assembling different media, and to avoid combining components that are incompatible upon autoclaving. Media are usually assembled from autoclaved or filter-sterilized stock solutions immediately before use, but most appear to be stable for extended periods in the refrigerator if contamination by mold spores is avoided. Stock solutions are stored at room temperature, except as noted in the recipes.

Fully defined, non-inducing media we currently use routinely: pages 6-7

MDAG-11 is a fully defined, non-inducing medium for agar plates and stabs to select, titer and distribute expression strains, and for liquid suspension of colonies to be retained temporarily as standing cultures at room temperature when purifying strains. MDG and MDAG-135 are fully defined, non-inducing media for growing shaking cultures of expression strains for freezer stocks, working stocks (which remain highly viable for weeks in the refrigerator), and for preparing plasmids from BL21(DE3) or other host strains susceptible to unintended induction.

Cultures that grow slowly because they are stressed by basal expression of a highly toxic target protein should be grown in MDAG-135 rather than MDG. Such cultures are likely to contain a mixture of newly-divided growing cells that have not yet produced a transcript of the toxic gene and cells that are dead or dying because they have produced a burst of toxic target protein, perhaps as little as that generated from a single transcript. The faster rate of division in MDAG-135 should increase the fraction live cells in the population and may allow the production of cultures where essentially all of the live cells are capable of producing target protein rather than consisting primarily of poorly expressing mutants that have overgrown the culture.

Auto-inducing media we currently use routinely: page 8

ZYM-5052 is a complex auto-inducing medium and MDA-5052 is a fully defined auto-inducing medium, both of which can support growth to relatively high densities and produce substantial amounts of a wide range of target proteins. Addition of arabinose to ZYM-5052 or MDA-5052 at a final concentration of 0.05% produces media for auto-induction of target genes in BL21-AI (Invitrogen), where production of T7 RNA polymerase is under control of the pBAD promoter and the target gene is expressed from the T7*lac* promoter.

Complex medium we currently use for rapid growth of high-density cultures: page 9

ZYM-505 is a complex medium that produces dense cultures of commonly used lab strains, satisfies complex nutritional requirements, and is useful for rapid growth of high-density cultures for preparing plasmids. The possibility of unintended induction may make ZYM-505 poorly suited for growing some expression strains.

Auto-inducing media for labeling target proteins with SeMet, ^{15}N or ^{13}C: pages 10-11

Low-phosphate (25 mM) non-inducing and auto-inducing minimal media: page 12

High-phosphate (100 mM) non-inducing and auto-inducing media used earlier: page 13-14

Stock solutions: pages 15-20

Non-inducing medium for agar plates and soft agar stabs

MDAG-11 plates (50 mM phosphate) non-inducing minimal medium +aa

MDAG-11 plates plus appropriate antibiotic(s) and any required nutritional supplement(s) are used to isolate transformants and to titer cells carrying expression plasmids. Colonies grow almost as well on fully defined MDAG-11 plates as on TBY plates, and at least some strains that express proteins toxic to the host make colonies on MDAG-11 but not on TBY plates. Stabs in MDAG soft agar (0.7%) are used for distributing expression strains.

To make 500 ml of 1% agar (~20 ml per plate yields ~25 plates)

5 g agar
~475 ml H_2O
autoclave 15 min, mix well, let cool ~10 min on bench

Add		Final composition	
1	ml 1 M $MgSO_4$	25 mM Na_2HPO_4	
100	µl 1000x metals	25 mM KH_2PO_4	
1.25	ml 40% glucose	50 mM NH_4Cl	
2	ml 25% aspartate	5 mM Na_2SO_4	
10	ml 50xM	2 mM $MgSO_4$	
14	ml 18aa (7.14 mg/ml each)	0.2x metals	= 10 µM Fe + 9
		0.1% glucose	= 5.6 mM
		0.1% aspartate	= 7.5 mM
		200 µg/ml each of 18 aa (no C,Y)	

Add other required nutrients or selective antibiotics

Mix well, pour ~20 ml per plate (slowly pouring into each standard plastic Petri plate until liquid just covers the bottom of the plate usually gives about the right amount per plate)

MDAG-11+B1+K plates (for routine use with kanamycin-resistant strains)

This recipe contains thiamine (B1), required for growth of XL1Blue-MR, and kanamycin for selection of expression plasmids. B1 is not needed by BL21(DE3).

Follow above MDAG-11 recipe; add the following after autoclaving the agar and before mixing to pour the plates

Add		Final composition	
50	µl 10 mM thiamine (B1)	1 µM thiamine (B1)	
2	ml kanamycin (25 mg/ml)	100 µg/ml kanamycin	

Non-inducing media used routinely for isolating expression strains and growing freezer stocks and seed cultures for auto-induction

MDAG-11 (50 mM phosphate) non-inducing for suspending colonies, dilution

To make 10 ml	Final composition	
9.43 ml H_2O	25 mM Na_2HPO_4	
20 μl 1M $MgSO_4$	25 mM KH_2PO_4	
2 μl 1000x metals	50 mM NH_4Cl	
25 μl 40% glucose	5 mM Na_2SO_4	
40 μl 25% aspartate	2 mM $MgSO_4$	
200 μl 50xM	0.2x metals	= 10 μM Fe + 9
280 μl 18aa (7.14 mg/ml each)	0.1% glucose	= 5.6 mM
	0.1% aspartate	= 7.5 mM
	200 μg/ml each of 18aa (no C,Y)	

MDG (50 mM phosphate) non-inducing minimal medium for shaking cultures

To make 10 ml	Final composition	
9.55 ml H_2O	25 mM Na_2HPO_4	
20 μl 1M $MgSO_4$	25 mM KH_2PO_4	
2 μl 1000x metals	50 mM NH_4Cl	
125 μl 40% glucose	5 mM Na_2SO_4	
100 μl 25% aspartate	2 mM $MgSO_4$	
200 μl 50xM	0.2x metals	= 10 μM Fe + 9
	0.5% glucose	= 27.8 mM
	0.25% aspartate	= 18.8 mM

MDAG-135 (50 mM phosphate) non-inducing minimal +aa for shaking cultures

To make 10 ml	Final composition	
9.37 ml H_2O	25 mM Na_2HPO_4	
20 μl 1M $MgSO_4$	25 mM KH_2PO_4	
2 μl 1000x metals	50 mM NH_4Cl	
87.5 μl 40% glucose	5 mM Na_2SO_4	
40 μl 25% aspartate	2 mM $MgSO_4$	
200 μl 50xM	0.2x metals	= 10 μM Fe + 9
280 μl 18aa(7.14 mg/ml each)	0.35% glucose	= 19.4 mM
	0.1% aspartate	= 7.5 mM
	200 μg/ml each of 18aa (no C,Y)	

Optional additions (per 10 ml):

1 μl 10 mM thiamine (B1)	1 μM thiamine (B1)
40 μl kanamycin (25 mg/ml)	100 μg/ml kanamycin

Auto-inducing media currently used routinely

ZYM-5052 (50 mM phosphate) auto-inducing complex medium

To make 10 ml	Final composition
9.57 ml ZY	1 % N-Z-amine AS
20 µl 1M $MgSO_4$	0.5% yeast extract
2 µl 1000x metals (optional)	25 mM Na_2HPO_4
200 µl 50x5052	25 mM KH_2PO_4
200 µl 50xM	50 mM NH_4Cl
	5 mM Na_2SO_4
	2 mM $MgSO_4$
	0.2x metals (optional)
	0.5% glycerol = 54 mM
	0.05% glucose = 2.8 mM
	0.2% α-lactose = 5.6 mM

MDA-5052 (50 mM phosphate) auto-inducing minimal medium +aa

Recipe differs from that given in the publication and previous handouts; it contains 0.1% aspartate instead of 0.25%, for better control of pH

To make 10 ml	Final composition
9.26 ml H_2O	25 mM Na_2HPO_4
20 µl 1M $MgSO_4$	25 mM KH_2PO_4
2 µl 1000x metals	50 mM NH_4Cl
200 µl 50x5052	5 mM Na_2SO_4
40 µl 25% aspartate	2 mM $MgSO_4$
200 µl 50xM	0.2x metals = 10 µM Fe + 9
280 µl 18aa (7.14 mg/ml each)	0.5 % glycerol = 54 mM
	0.05% glucose = 2.8 mM
	0.2 % α-lactose = 5.6 mM
	0.1% aspartate = 18.8 mM
	200 µg/ml each of 18aa (no C,Y)

Optional additions (per 10 ml):

1 µl 10 mM thiamine (B1)	1 µM B1
40 µl kanamycin (25 mg/ml)	100 µg/ml kanamycin
20 µl 1000x metals	1x metals = 50 µM Fe + 9 individual metal salts for known metal-binding target proteins

Media with arabinose for auto-induction from T7*lac* in BL21-AI

Add arabinose to the any of the auto-inducing media to give a final concentration of 0.05% arabinose

Auto-inducing minimal medium

MD-5052 (50 mM phosphate) auto-inducing minimal medium

To make 10 ml	Final composition	
9.48 ml H_2O	25 mM Na_2HPO_4	
20 µl 1M $MgSO_4$	25 mM KH_2PO_4	
2 µl 1000x metals	50 mM NH_4Cl	
200 µl 50x5052	5 mM Na_2SO_4	
100 µl 25% aspartate	2 mM $MgSO_4$	
200 µl 50xM	0.2x metals	= 10 µM Fe + 9
	0.5 % glycerol	= 54 mM
	0.05% glucose	= 2.8 mM
	0.2 % α-lactose	= 5.6 mM
	0.25% aspartate	= 18.8 mM

Complex medium for rapid growth of high-density cultures

ZYM-505 (50 mM phosphate) complex medium, possible unintended induction

This medium is used for rapid growth of high-density cultures for preparing plasmids from strains that do not supply T7 RNA polymerase, and sometimes for growing strains that express innocuous target proteins. However, the possibility of unintended induction may make this medium poorly suited for growing some expression strains, particularly those in which the target protein may stress the host. Plasmids from T7 expression strains are usually isolated from cultures grown on fully defined non-inducing media such as MDG or MDAG.

To make 10 ml	Final composition
9.68 ml ZY	1 % N-Z-amine AS
20 µl 1M $MgSO_4$	0.5% yeast extract
2 µl 1000x metals (optional)	25 mM Na_2HPO_4
100 µl 100x505	25 mM KH_2PO_4
200 µl 50xM	50 mM NH_4Cl
	5 mM Na_2SO_4
	2 mM $MgSO_4$
	0.2x metals (optional)
	0.5% glycerol = 54 mM
	0.05% glucose = 2.8 mM

add appropriate selective antibiotic(s)

Auto-inducing medium for labeling with SeMet

Greater than 90% replacement of methionines by SeMet in target proteins auto-induced in this medium was found whether using the prototroph BL21(DE3) or the methionine auxotroph, B834 (*metE*), apparently because the presence of Met/SeMet in the medium represses the methionine-synthesis pathway. Yields of SeMet-labeled target protein in PASM-5052 have been comparable to yields obtained by auto-induction in the absence of SeMet. In PASM-5052, unlabeled methionine at 10 μg/ml facilitates growth and auto-induction in 125 μg/ml of SeMet, which would otherwise be too toxic to allow high-density growth and auto-induction. Vitamin B_{12} stimulates the *E. coli metH* enzyme to catalyze regeneration of SeMet from the selenohomocysteine generated in methylation reactions. Growth at 37°C from a thousand-fold dilution typically reaches saturation in 14-16 hr. Growth at 20°C is much slower and a culture can take 3 days or longer to become induced and reach saturation. PASM-5052 contains 100 mM phosphate and was based on PA-5052 (page 14) before development of lower phosphate auto-inducing media; it could presumably be reformulated as MDASM-5052, containing 50 mM total phosphate and 0.1% aspartate, based on MDA-5052 (page 8).

PASM-5052 (100 mM phosphate) auto-inducing minimal medium +aa

To make 10 ml	Final composition
9.01 ml H_2O	50 mM Na_2HPO_4
20 μl 1M $MgSO_4$	50 mM KH_2PO_4
2 μl 1000x metals	25 mM $(NH_4)_2SO_4$
200 μl 50x5052	2 mM $MgSO_4$
500 μl 20xP	0.2x metals = 10 μM Fe + 9
200 μl 17aa(10 mg/ml each)	0.5 % glycerol = 54 mM
4 μl Met (25 mg/ml)	0.05% glucose = 2.8 mM
50 μl SeMet (25 mg/ml)	0.2 % α-lactose = 5.6 mM
10 μl 100 μM vitamin B_{12}	200 μg/ml each of 17aa (no C,Y,M)
	10 μg/ml Met
	125 μg/ml SeMet
	100 nM vitamin B_{12}

To make 800 ml

725 ml H_2O
1.6 ml 1M $MgSO_4$
160 μl 1000x metals
16 ml 50x5052
40 ml 20xP
16 ml 17aa(10 mg/ml each)
320 μl Met (25 mg/ml)
100 mg bottle of SeMet
800 μl 100 μM vitamin B_{12}

Auto-inducing minimal media for labeling with ^{15}N and ^{13}C

Target proteins may be labeled in auto-inducing media in which the sole nitrogen source is $^{15}NH_4Cl$ (LS-5052, page 12, or N-5052, below) or $(^{15}NH_4)_2SO_4$ (P-5052, page 14). The 50 mM $^{15}NH_4$ in these media supports growth to high density, and high levels of expression make efficient use of the label. Reducing ammonium concentration below ~25 mM may significantly limit target protein expression in these media.

N-5052 (100 mM phosphate) auto-inducing minimal medium for ^{15}N labeling

Final composition

50 mM Na_2HPO_4
50 mM KH_2PO_4
50 mM $^{15}NH_4Cl$
5 mM Na_2SO_4
2 mM $MgSO_4$
0.2x metals = 10 μM Fe + 9
0.5 % glycerol = 54 mM
0.05% glucose = 2.8 mM
0.2 % α-lactose = 5.6 mM

Target proteins may be labeled with ^{13}C-glycerol by auto-induction in C-750501 medium. The glycerol concentration has been increased to 0.75% and the lactose concentration decreased to 0.01% to minimize the flow of carbon from lactose into target protein (glucose is depleted before target protein is induced). The 0.01% lactose will give good induction if aeration is not too high. This medium is an initial formulation aiming for maximum incorporation, and a systematic analysis may find that satisfactory labeling can be achieved at lower concentrations of ^{13}C-glycerol, higher concentrations of lactose and higher rates of aeration. The small amount of glucose could also be labeled with ^{13}C relatively inexpensively.

C-750501(100 mM phosphate) auto-inducing minimal medium for ^{13}C labeling

Final composition

50 mM Na_2HPO_4
50 mM KH_2PO_4
50 mM NH_4Cl
5 mM Na_2SO_4
2 mM $MgSO_4$
0.2x metals = 10 μM Fe + 9
0.75 % ^{13}C-glycerol = 81 mM
0.05% glucose = 2.8 mM
0.01 % α-lactose = 0.28 mM

Low-phosphate (25 mM) non-inducing and auto-inducing minimal media

Succinate provides metabolic control of pH in these media, but aspartate could also be used. Phosphate becomes limiting for growth to high density if its concentration is reduced much below 25 mM.

LSG (25 mM phosphate) non-inducing minimal medium
(previously referred to as NIMS)

To make 10 ml	Final composition	
9.45 ml H_2O	12.5 mM Na_2HPO_4	
20 µl 1M $MgSO_4$	12.5 mM KH_2PO_4	
2 µl 1000x metals	50 mM NH_4Cl	
125 µl 40% glucose	5 mM Na_2SO_4	
200 µl 1M succinate	2 mM $MgSO_4$	
200 µl 50xL	0.2x metals	= 10 µM Fe + 9
	0.5% glucose	= 27.8 mM
	20 mM succinate	= 0.23%

LS-5052 (25 mM phosphate) auto-inducing minimal medium

To make 10 ml	Final composition	
9.38 ml H_2O	12.5 mM Na_2HPO_4	
20 µl 1M $MgSO_4$	12.5 mM KH_2PO_4	
2 µl 1000x metals	50 mM NH_4Cl	
200 µl 50x5052	5 mM Na_2SO_4	
200 µl 1 M succinate	2 mM $MgSO_4$	
200 µl 50xL	0.2x metals	= 10 µM Fe + 9
	0.5 % glycerol	= 54 mM
	0.05% glucose	= 2.8 mM
	0.2 % α-lactose	= 5.6 mM
	20 mM succinate	= 0.23%

Earlier non-inducing media containing 100 mM phosphate

PG (100 mM phosphate) non-inducing minimal medium
(previously referred to as P-0.5G)

To make 10 ml	Final composition	
9.35 ml H_2O	50 mM Na_2HPO_4	
20 µl 1M $MgSO_4$	50 mM KH_2PO_4	
2 µl 1000x metals	25 mM $(NH_4)_2SO_4$	
125 µl 40% glucose	2 mM Mg_2SO_4	
500 µl 20xP	0.2x metals	= 10 µM Fe + 9
	0.5% glucose	= 27.8 mM

PAG (100 mM phosphate) non-inducing minimal medium +aa

To make 10 ml	Final composition	
9.07 ml H_2O	50 mM Na_2HPO_4	
20 µl 1M $MgSO_4$	50 mM KH_2PO_4	
2 µl 1000x metals	25 mM $(NH_4)_2SO_4$	
125 µl 40% glucose	2 mM Mg_2SO_4	
500 µl 20xP	0.2x metals	= 10 µM Fe + 9
280 µl 18aa(7.14 mg/ml each)	0.5% glucose	= 27.8 mM
	200 µg/ml each of 18aa (no C,Y)	

Earlier auto-inducing media containing 100 mM phosphate

ZYP-5052 (100 mM phosphate) auto-inducing complex medium
(previously used routinely)

To make 10 ml	Final composition
9.28 ml ZY	1 % N-Z-amine AS
20 µl 1M $MgSO_4$	0.5% yeast extract
2 µl 1000x metals	50 mM Na_2HPO_4
200 µl 50x5052	50 mM KH_2PO_4
500 µl 20xP	25 mM $(NH_4)_2SO_4$
	2 mM $MgSO_4$
	0.2x metals = 10 µM Fe + 9
	0.5% glycerol = 54 mM
	0.05% glucose = 2.8 mM
	0.2% α-lactose = 5.6 mM

PA-5052 (100 mM phosphate) auto-inducing minimal medium +aa

To make 10 ml	Final composition
9.00 ml H_2O	50 mM Na_2HPO_4
20 µl 1M $MgSO_4$	50 mM KH_2PO_4
2 µl 1000x metals	25 mM $(NH_4)_2SO_4$
200 µl 50x5052	2 mM $MgSO_4$
500 µl 20xP	0.2x metals = 10 µM Fe + 9
280 µl 18aa(7.14 mg/ml each)	0.5 % glycerol = 54 mM
	0.05% glucose = 2.8 mM
	0.2 % α-lactose = 5.6 mM
	200 µg/ml each of 18aa (no C,Y)

P-5052 (100 mM phosphate) auto-inducing minimal medium

To make 10 ml	Final composition
9.58 ml H_2O	50 mM Na_2HPO_4
20 µl 1M $MgSO_4$	50 mM KH_2PO_4
2 µl 1000x metals	25 mM $(NH_4)_2SO_4$
200 µl 50x5052	2 mM $MgSO_4$
200 µl 50xM	0.2x metals = 10 µM Fe + 9
	0.5 % glycerol = 54 mM
	0.05% glucose = 2.8 mM
	0.2 % α-lactose = 5.6 mM

Stock solutions

Use deionized distilled water for all solutions
Autoclave solutions for 15 min unless specified otherwise
Dissolve sequentially in water stirred in beaker
Dissolve to completion; if necessary, heat in microwave
(plastic-covered magnetic stirring bars need not be removed)

50xL (1xL = 25 mM PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4)

To make 100 ml

	MW	50x	1x
85 ml H_2O			
8.875 g Na_2HPO_4	142.0	0.625 M	12.5 mM
8.5 g KH_2PO_4	136.1	0.625 M	12.5 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

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_2O			
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 microwave

20xP (1xP = 100 mM PO_4, 25 mM $(NH_4)_2SO_4$)

To make 100 ml

	MW	20x	1x
90 ml H_2O			
14.2 g Na_2HPO_4	142.0	1.0 M	50 mM
13.6 g KH_2PO_4	136.1	1.0 M	50 mM
6.6 g $(NH_4)_2SO_4$	132.1	0.5 M	25 mM

pH of 50-fold dilution should be ~6.7

Stock solutions

Use deionized distilled water for all solutions
Autoclave solutions for 15 min unless specified otherwise
Dissolve sequentially in water stirred in beaker
Dissolve to completion; if necessary, heat in microwave
(plastic-covered magnetic stirring bars need not be removed)

1 M $MgSO_4$

24.65 g $MgSO_4$-$7H_2O$
87 ml H_2O

40% glucose (w/v)

To make 100 ml

74 ml H_2O
40 g glucose

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

100 g glycerol (weigh in beaker)
20 ml H_2O

50x5052 (1x5052 = 0.5% glycerol, 0.05% glucose, 0.2% α-lactose)

To make 100 ml

25 g glycerol (weigh in beaker)
73 ml H_2O
2.5 g glucose
10 g α-lactose monohydrate

100x505 (1x505 = 0.5% glycerol, 0.05% glucose)

To make 100 ml

50 g glycerol (weigh in beaker)
57 ml H_2O
5 g glucose

Stock solutions

Use deionized distilled water for all solutions
Autoclave solutions for 15 min unless specified otherwise
Dissolve sequentially in water stirred in beaker
Dissolve to completion; if necessary, heat in microwave
(plastic-covered magnetic stirring bars need not be removed)

ZY

10 g N-Z-amine AS (or tryptone, other enzymatic digests of casein)
5 g yeast extract
1 liter of H_2O

25% aspartate (sodium salt)

	MW	Concentration
84 ml H_2O		
25 g aspartic acid	133	1.88 M
8 g NaOH	40	2.0 M

pH of final solution should be in the neutral range

1 M succinate (disodium salt)

	MW	Concentration
27 g disodium succinate - $6H_2O$	270.1	1 M
to 100 ml with H_2O		
or		
11.8 g succinic acid	118.1	1 M
~8 g NaOH	40.0	2 M
to 100 ml with H_2O		

pH of final solution should be in the neutral range

1 M citrate (trisodium salt)

	MW
29.4 g trisodium citrate dihydrate	294.1
H_2O to make 100 ml	

100 μM vitamin B_{12} (cyanocobalamin)

	MW
13.55 mg vitamin B_{12}	1355
100 ml autoclaved H_2O	
Filter sterilize, store in refrigerator	

10 mM thiamine (vitamin B1)

	MW
337.3 mg thiamine HCl	337.27
100 ml autoclaved H_2O	
Filter sterilize, store in refrigerator	

Amino-acid stock solutions

Methionine	MW	Concentration
25 mg/ml Autoclave 15 min	149.2	168 mM

17aa (no C,Y,M) (10 mg/ml each)

To make 100 ml

90 ml H_2O stirred in beaker
Add 1 g each:

			MW	Concentration
1	E	Na Glu	169.1	59 mM
2	D	Asp	133.1	75 mM
3	K	Lys-HCl	182.6	55 mM
4	R	Arg-HCl	210.7	47 mM
5	H	His-HCl-H_2O	209.6	48 mM
6	A	Ala	89.09	112 mM
7	P	Pro	115.1	87 mM
8	G	Gly	75.07	133 mM
9	T	Thr	119.1	84 mM
10	S	Ser	105.1	95 mM
11	Q	Gln	146.1	68 mM
12	N	Asn-H_2O	150.1	67 mM
13	V	Val	117.1	85 mM
14	L	Leu	131.2	76 mM
15	I	Ile	131.2	76 mM
16	F	Phe	165.2	61 mM
17	W	Trp	204.2	49 mM

Stir until everything dissolves
If necessary, heat in microwave
Filter sterilize, store in refrigerator

The mixture of 17 amino acids is quite acidic and may have to be neutralized when using final concentrations higher than 200 μg/ml of each amino acid. The effect of neutralizing the stock solution has not been explored. Trp and His slowly oxidize, producing a slightly yellow color. Tyr and Cys are not included because Tyr has low solubility and Cys oxidizes with time to precipitate as insoluble cystine.

18 aa (no C,Y) (7.14 mg/ml each)	Total aa concentration
10 ml 17 aa (10 mg/ml each)	960 mM
4 ml methionine (25 mg/ml)	48 mM

280 μl of 18aa in a total of 10 ml medium gives 200 μg/ml each

All 18 amino acids could be dissolved together at 10 mg/ml of each but the solution did not remain soluble upon storage in the refrigerator.

Trace metals

0.1 M $FeCl_3$ in ~0.12 M HCl

	MW
99 ml sterile H_2O	
1 ml conc HCl (~12 M)	
2.7 g $FeCl_3$-$6H_2O$	270.30

Do not autoclave, as a large precipitate forms

1000x trace metals mixture

Used at 0.2x to satisfy requirements for growth, or at 1x to attempt to supply metals to target proteins of unknown metal-binding potential

To make 100 ml

		MW	1x conc
36 ml	sterile H_2O		
50 ml	0.1 M $FeCl_3$ in ~0.12 M HCl	270.30	50 μM Fe
2 ml	1 M $CaCl_2$	110.99	20 μM Ca
1 ml	1 M $MnCl_2$-$4H_2O$	197.91	10 μM Mn
1 ml	1 M $ZnSO_4$-$7H_2O$	287.56	10 μM Zn
1 ml	0.2 M $CoCl_2$-$6H_2O$	237.95	2 μM Co
2 ml	0.1 M $CuCl_2$-$2H_2O$	170.486	2 μM Cu
1 ml	0.2 M $NiCl_2$-$6H_2O$	237.72	2 μM Ni
2 ml	0.1 M Na_2MoO_4-$2H_2O$	241.98	2 μM Mo
2 ml	0.1 M Na_2SeO_3-$5H_2O$	263.03	2 μM Se
2 ml	0.1 M H_3BO_3	61.83	2 μM B

Except for $FeCl_3$, stock solutions of individual metals were autoclaved and solutions were stored at room temperature. 1000x metals tended to form a slight precipitate with time.

Lack of trace metals limited growth in fully defined media but not in complex ZY-containing media. However, the trace metal content of ZY might well vary from lot to lot, and addition of 0.2x metals assures that any metals requirement would be met.

The 1x trace metals mixture is an attempt to saturate most metal-binding target proteins, but 0.2x is sufficient to support growth to high culture densities. As much as 5x can be tolerated with little effect on saturation density. Cobalt and selenate are tolerated at 10 μM but not 100 μM; the other metals are tolerated to at least 100 μM.

The greatest need is for iron. Less than 5 μM iron limited growth in minimal media and less than 10 μM iron limited growth in defined media containing amino acids. If a trace metal mixture is not available, 100 μM iron supported growth in a defined medium almost as well as the full trace metals mixture. The highest iron concentration tested, 800 μM $FeCl_3$, remained soluble in 1 mM citrate and was well tolerated.

Antibiotic stock solutions

Selective antibiotics are used for growth of expression strains in non-inducing media, to ensure that almost all cells in freezer stocks and working cultures carry plasmids capable of inducing target protein. However, most expression strains retain plasmid so well in non-inducing media, have such low levels of expression of target protein in the early stages of growth in auto-inducing media, and auto-induce so close to saturation that the presence of selective antibiotics may not be necessary for reliable, high-level expression of most target proteins in auto-inducing media.

Ampicillin

50 mg/ml in H_2O, stored in -20°C freezer
Used at 50 μg/ml

Chloramphenicol

25 mg/ml in ethanol, stored in refrigerator or freezer
Used at 25 μg/ml

Kanamycin

25 mg/ml in H_2O, stored in refrigerator
Used at 100 μg/ml

BL21(DE3) is sensitive to kanamycin in LB liquid culture or plates, failing to grow in as little as 10 μg/ml. However, high concentrations of phosphate, together with amino acids, were found to increase resistance to kanamycin. BL21(DE3) containing no plasmid to confer resistance will grow, albeit somewhat slowly, in ZYP-5052 (100 mM PO_4) containing 100 μg/ml of kanamycin and is killed effectively only at concentrations in the range of 400 μg/ml, and the cells that grow have normal sensitivity to kanamycin when tested on LB plates. BL21(DE3) also grows reasonably well in commonly used media such as M9TB (64 mM PO_4) or terrific broth (89 mM PO_4) containing 25 μg/ml of kanamycin. The non-inducing and auto-inducing media having 25 or 50 mM phosphate were developed to provide greater sensitivity to kanamycin. A concentration of 100 μg/ml of kanamycin provides satisfactory killing in such media.