PROZOMIX
Recombinant Enzymes and Proteins

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"FIFTYOD^{TM}" High Cell Density Growth Medium Protocol

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Cat. No.:	**PRO-FIFTYOD-2L (trial pack to make total of 2 L medium)**
Contents:	4 x 49 g FIFTYOD paste 1 x 0.15 mL antifoam (sterile) 1 x protocol

!!! PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING YOUR EXPERIMENT !!!

NOTE: this protocol is tailored specifically for *E. coli* BL21 along with the 49 g (0.5 L) trial pack size of FIFTYOD^{TM}, and should be considered as a starting point only. On average, if the protocol is followed carefully, FIFTYOD will yield approx. the same amount of soluble recombinant protein per optical density (OD) unit, as is achieved using LB medium. However, as the ODs achieved with FIFTYOD are approx. 20 x greater than for LB, the final yield of recombinant protein will be approx. 20 x higher per L! Thus it is possible to estimate with some accuracy how much recombinant protein will be produced from your historical expression results. Like for all protein expression experiments, once suitability has been established, optimisation can often be possible, by systematically changing common key parameters, i.e. growth temperatures before and after induction, type and concentration of inducing agent, target density for induction, length of incubation post-induction, volume of medium per flask, and other "tricks" you may normally employ, etc. Please note carefully, that the trajectory of optical density development described in this protocol is generally experienced, but certain recombinant proteins, especially enzymes, can significantly delay onset of rapid growth. In some cases growth can actually lag for 1-2 days, and thus cultures that are not showing signs of growth should be left on the shaker to enable metabolic acclimatisation to occur. Such a phenomenon only occurs approx. 15 % of the time (even when working solely with recombinant enzymes), and the eventual growth generally results in the expected yield of soluble protein per OD unit.

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"FIFTYOD™" High Cell Density Growth Medium Protocol

DAY ONE:

1. 09:00 to 10:00 – using a 28 mL Sterilin® plastic container, or equivalent glass or plastic container, inoculate 5 mL of LB liquid growth medium (or your normal / general low-density growth medium), with the equivalent of ½ an inoculating loop full of your expressing *E. coli* strain (from an agar plate that is less than 2 weeks old). Add your antibiotic as usual and incubate with shaking at approx. 140 rpm and 25°C. Take a FIFTYOD trial pack (49 g) out of the freezer and allow to equilibrate to room temperature. Then using a laboratory spoon or (sturdy) spatula, remove approx. 20 % of the medium as a ball shape, and allow it to drop off the spoon / spatula, directly into a 2 L baffled flask (4 x vertical side baffles works best, these can be purchased from Prozomix for 30 GBP each if necessary, and each of these flasks has been tested to ensure minimum splashing at the optimum speed of 140 rpm). Repeat until the majority of the FIFTYOD paste is in the 2 L flask. Place the 2 L flask on a balance and zero. Add approx. 50 mL deionised (or better) water to the plastic container that contained the FIFTYOD paste, screw the lid on, and shake vigorously for 30 s to remove the residual medium. Pour the liquid into the 2 L flask. Wash the residual FIFTYOD paste out of the plastic container as above until it has all dissolved and has been removed to the flask. Now add water directly to the 2 L flask, preferably using a "squeezy" bottle, in order to wash any medium round the neck of the flask into the bottom of the flask, and to increase the weight of added water to 480 g (i.e. 480 mL). The volume in the flask is now approx. 505 mL.

NOTE: the balls of medium will be floating on the surface of the medium at this stage, do not try to dissolve fully, as this takes place very efficiently during autoclaving. The risk is that splashing onto the filter (described next) will cause clogging of the paper, and result in poor oxygen transfer during the critical early stages of fermentation.

The enclosure of the 2 L baffled flask is critical - the following should be followed PRECISELY:

Fold a single sheet (2-ply) of good quality laboratory white tissue paper in half, and then in half again. Place the approx. square (now 8-ply) paper "filter" symmetrically on the mouth of the flask and secure with a strip of autoclave tape (all the way around). Autoclave the prepared 2 L baffled flask containing the 505 mL of FIFTYOD as per your normal sterilisation protocol.

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2. 16:30 to 17:30 – using excellent aseptic technique, carefully remove 5 mL of sterile FIFTYOD from the 2 L flask and add it directly to the 5 mL culture prepared as in **1.** above. There is no need to add additional antibiotic or any other additive(s) at this stage, during which the bacterial cells take many hours to metabolically adapt to the new medium components, and will divide only a few times. Incubate with the lid screwed shut with shaking at approx. 140 rpm overnight at 25°C (do not incubate at higher temperatures, as the cells may approach/enter the stationary phase, and this can result in very poor fermentation).

DAY TWO:

1. 09:00 to 10:00 - inoculate the 2 L baffled flask containing (now) 500 mL FIFTYOD liquid growth medium by pouring in the entire 10 mL culture from DAY ONE step 2 above. Add your antibiotic as usual, along with 0.025 mL of the antifoam provided (this is very viscous, but use of a 0.2 mL pipette with a tip that has had the very end cut-off is sufficient, with a slow / careful pipetting action to ensure accuracy. This addition is made easier if the widened-bore pipette tip is touched on the top of one of the 4 baffles while pipetting the antifoam into the flask, for example. Alternatively it should be added to the surface of the medium by tilting the flask. DO NOT ADD ABOVE THE MEDIUM LEVEL AS IT WILL NOT RUN DOWN TO THE MEDIUM).

NOTE: do not add the antifoam before autoclaving the medium, as it is not stable in diluted form to heat treatment.

It is recommended that the first time FIFTYOD is used, induction is achieved by IPTG (not provided in the kit) when the optical density is between 5 and 20 (as described below). This also enables animal-free IPTG to be used if necessary for quality purposes. However, if auto-induction is desired in subsequent experiments, at this stage immediately also add 2 mL 50 % (w/v) glucose (not provided in the kit) and 20 mL 20 % (w/v) lactose (not provided in the kit) by either filter sterilisation directly into the flask, or using your normal method (the resultant dilution of the medium, especially by the addition of the lactose, will not affect performance, and should not be accounted for in any way). If bacteriophage are currently present, or suspected of being present in the laboratory (NOTE: BL21 infective phage are present in probably all busy labs!!!), incubate the glucose and lactose solutions in a boiling water bath for 30 min and use with or without filter sterilisation, according to your normal practice / preference. If induction with IPTG worked in your first experiment, in subsequent experiments it may be optimal to add 2 mL 50 % (w/v) glucose at this stage (as this promotes initial / rapid onset of bacterial growth in non-expressing state, enabling the

culture to establish healthy growth more reliably in general).

Incubate the 2 L baffled flask at just 140 rpm and at 25°C (or ambient if temperature control is not available).

2. 17:00 to 17:30 - inspect the flask, growth may now be visually apparent but the OD will be low, at approx. 0.5-2, this is normal. There is no need to measure the optical density at this stage (in general with some practice / experience the optical density can be estimated fairly accurately throughout fermentation using FIFTYOD, as bacterial cells at densities > 10 have very characteristic optical characteristics, that are easy to appreciate (with experience) by swirling the flask and holding it up to a common laboratory light, etc. The medium colour also changes from dark brown, through pale brown, to almost a creamy white at very high ODs). **NOTE: frequent / unnecessary accurate measurement of optical densities significantly increases the risk of bacteriophage infection, especially in busy labs!!!).**

DAY THREE:

1. 09:00 to 10:00 - inspect the flask, growth will now (generally) be very apparent, with an OD approx. 5-20, this is normal. Take an accurate OD reading (against a water blank) by adding 0.03 mL culture to 2.97 mL water and reading at 600 nm in a 3 mL plastic cuvette (i.e. multiply your absorbance reading by 100 to get your OD). NOTE: bacterial cells in FIFTYOD sediment immediately at all optical densities, and thus you must agitate the flask well just prior to removing the 0.03 mL aliquot). Working rapidly to ensure a minimum period of time that the flask is not being agitated, also add 0.50 mL 1 M IPTG where IPTG was selected as inducer.

NOTE: it is now necessary to increase the flux of oxygen to the culture.

In order to achieve this, simply remove the 8-ply filter and replace with a flame sterilised large square (approx. dimensions as the base of the 2 L flask) of aluminium foil. NOTE: crimp the foil to allow good transfer of gases and apply very lightly to the neck of the flask, so lightly that the foil actually moves a little during shaking at 140 rpm. This step **IS ABSOLUTELY ESSENTIAL** at this stage of the protocol to ensure optimal aeration of the culture in the high-density phase of growth / recombinant protein expression. If this step is not performed effectively, optical density and protein expression levels will be reduced by at least 50 %!!!

2. 16:30 to 17:30 - take another OD reading and take a 0.3 mL sample for SDS-PAGE analysis on Day 4 (spin this sample down and remove the supernatant, as lysis may occur whilst being stored for analysis on Day 4, or alternatively, process to cell-free extract immediately; use your normal methods to prepare the samples for SDS-PAGE, adjusting the volume of culture used based on the OD of the FIFTYOD culture, compared to a typical OD for your usual lower density medium, i.e. LB, etc). At this stage the OD will be in the range of approx. 20-40. After taking the reading / sample, ensure the foil is replaced loose as described above, and continue incubation.

DAY FOUR:

1. 09:00 to 10:00 - take another OD reading and take an additional 0.3 mL sample of culture for SDS-PAGE analysis. At this stage the OD will be in the range of approx. 20-80. Analyse the samples to determine optimal point of soluble / functional recombinant protein expression for future reference. At this stage the bacterial cells can be processed as per your usual methods. Practical *TIP* - if a large volume of FIFTYOD is involved, combine the flasks into a smaller number of completely full flasks. Place these flasks in ice water (or cold tap water if necessary) for 2 hours or longer, and then carefully decant the supernatant off the settled cells. The longer the flasks are left, the better the sedimentation will be. Frequently the recombinant protein will be stable enough to allow convenient over-night sedimentation.

FINAL WARNING - the weight of cell pellet and the amount of soluble recombinant protein of interest will generally both scale in line with optical density. Thus if you normally process 0.5 L of cells from LB growth medium at a final OD of 3, and you then begin to process 0.5 L of cells grown in FIFTYOD to an OD of 60, you will have approx. 20 X more cell paste to process, and recombinant protein to isolate. This can lead to a very late evening in the lab for the unprepared!

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FIFTYOD RECORD SHEET								
Protein	Date / time	OD (600nm)	Additions / observations		Protein	Date / time	OD (600nm)	Additions / observations

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