SUMOpro-3® Gene Fusion Technology

NEW METHODS FOR ENHANCING FUNCTIONAL PROTEIN EXPRESSION AND PURIFICATION IN YEAST

Pichia pastoris Expression System

Cat. No. 2150 (Kit)
2151 (Vector)

Product Manual

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Background

Ubiquitin and SUMO

In cells, proteins are tagged for degradation by ubiquitin and sent to the 26S proteasome. In contrast, covalent modification of cellular proteins by the ubiquitin-like modifier SUMO (small ubiquitin-like modifier) regulates various cellular processes, such as nuclear transport, signal transduction, and stabilization of proteins. Ubiquitin-like proteins fall into two classes: the first class, ubiquitin-like modifiers (UBLs) function as modifiers in a manner analogous to that of ubiquitin. Examples of UBLs are SUMO, Rub1 (also called Nedd8), Apg8 and Apg12. Proteins of the second class include parkin, RAD23 and DSK2, are designated ubiquitin-domain proteins (UDPs). These proteins contain domains that are related to ubiquitin but are otherwise unrelated to each other. In contrast to UBLs, UDPs are not conjugated to other proteins. Once covalently attached to cellular targets, SUMO regulates protein:protein and protein:DNA interactions, as well as localization and stability of the target protein. Sumoylation occurs in most eukaryotic systems, and SUMO is highly conserved from yeast to humans. SUMO has an apparent molecular weight of ~12kDa. SUMO and ubiquitin only show about 18% homology, but both possess a common three-dimensional structure characterized by a tightly packed globular fold with β-sheets wrapped around an α-helix.

SUMO3 Fusions

Human SUMO3 fused with a protein-of-interest can enhance expression and promote solubility and correct folding of its fusion partner. It has been known for a long time that ubiquitin exerts chaperoning effects on fused proteins in E. coli and yeast, increasing their yield and solubility. Attachment of a highly stable structure (such as that of ubiquitin or SUMO) at the N-terminus of a partner protein increases the yield by increasing stability. The solubilizing effect of ubiquitin and ubiquitin-like proteins may also be explained in part by the outer hydrophilicity and inner hydrophobicity of the core structure of ubiquitin and SUMO, exerting a detergent-like effect on otherwise insoluble proteins.

Purification and SUMO Protease 2

While ubiquitin fusion has been known for many years to enhance protein expression, its utility as a protein purification modality is compromised by the inefficient nature of ubiquitin hydrolase, or protease – the enzyme that cleaves the partner protein from ubiquitin by hydrolysing the peptide bond. Likewise, commonly used proteases such as thrombin, enterokinase, rhinovirus proteases, and TEV, do not cleave all fusions efficiently and, moreover, can generate unnatural N-termini by leaving intact some amino acids from the cleavage recognition site. In eukaryotic cells ubiquitin is not a convenient tag since ubiquitinated proteins are a target for the degradation machinery.

Recently, SUMO Protease 2 (SENP2 core), a Human SUMO protease equivalent of ubiquitin protease, has been evaluated as a tool for purification of proteins expressed in *E. coli* and is extremely efficient for cleavage of the human SUMO3 tag. SUMO Protease 2 recognizes the tertiary structure of human SUMO3 at the N-terminus of the fusion partner protein and cleaves the junction irrespective of the N-terminal sequence of the protein (with the exception of proline). The accuracy and efficiency of SUMO Protease 2 cleavage is excellent and never cleaves within the protein-of-interest.

Benefits

The benefits of the SUMOpro®-3 Expression System:

1) Human SUMO3 fusion often enhances expression.

2) Human SUMO3 fusion often enhances protein solubility.

3) No known case of SUMO Protease 2 cleaving within the fused protein-of-interest.

4) Cleavage yields native protein with a desired N-terminus.

Components

The Pichia hSUMO3 Expression System provides the reagents to express a protein of interest as a fusion construct with the hSUMO3 protein tag. The system contains the following five components:

1) pP-αhSUMO3 (Zeo, pAOX1)
Size: 20µg (0.5µg/µl)
Buffer: 10mM Tris

2) SUMO Protease 2
Size: 500 units
10 Units/µL, 50 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM DTT, 1 mM EDTA

3) SUMO Protease 2 Control Protein
Size: 100µg (10µg/µl)
Buffer: PBS

4) Chicken Anti – hSUMO3
Size: 50µg (0.5mg/ml)
Buffer: 25 mM Tris- HCl, pH 7.5
150mM NaCl

Storage

pP-αhSUMO3 Vector (Zeo, pA0X1)
Store vial at -20°C.

SUMO Protease 2
Store vial at -80°C. Avoid cycles of freezing and thawing.

SUMO Protease 2 control protein
Store vial at -80°C. Avoid cycles of freezing and thawing.

Anti–hSUMO3
Store vial at -20°C prior to use. For extended storage aliquot contents and freeze at -20° C or below. Avoid cycles of freezing and thawing. Centrifuge product if not completely clear after standing at room temperature. This product is stable for several weeks at 4°C as an undiluted liquid. Dilute only prior to immediate use.

Cloning

Background

The pP-αSUMO3 vector is provided as a circular plasmid. For cloning, the vector has to be digested with BsmBI restriction endonuclease. This digest will drop out a small fragment and leaving two unique overhangs; ACCT at the 5' end and a CTAG (XbaI) overhang at the 3'. Two different overhangs allow directional insertion of the gene of interest (see Multiple Cloning Site (MCS) map). The ACCT at the end of the SUMO3 coding sequence, allows a gene of interest to be cloned in frame with the hSUMO3 tag, resulting in a SUMO3 fusion protein construct.

Forward Primer Design

To clone your gene of interest into the pP-αSUMO3 vector, it must be amplified by PCR and digested to produce an overhang complementary to the vector's ACCT. This can be accomplished by way of Class IIS restriction enzymes, which recognize non-palindromic sequences and cleave at sites that are outside their recognition sequences. The latter trait gives Class IIS enzymes two useful properties. First, when a Class IIS enzyme recognition site is engineered at the end of a primer, the site is removed from the PCR product when it is digested, meaning that there will be no extraneous sequence between SUMO3 and your gene. Second, overhangs created by Class IIS enzymes are template-derived and thus unique.

Below is an example of forward primer design incorporating a restriction site for the Class IIS enzyme BsaI:

Gene Target

BsmBI: 5' – **GGTCTC**NAGGTXXXXXXXXXXXXXXX – 3'

Where **CGTCTC** is the BsmBI recognition sequence, N is any nucleotide, AGGT will be the overhang that is complementary with the ACCT end of the pP-αSUMO3 vector. XXX is the first codon of your gene of interest and GGT is the last codon of the SUMO3 tag. Additional nucleotides will be required for the primer to anneal specifically with your gene of interest during the PCR amplification.

If your gene of interest already contains a BsmBI site, then another Class IIS enzyme and site may be used instead. Below are examples of forward primers for some of these enzymes/sites:

AarI 5' - **CACCTGC**NNNNAGGTXXXXXXXXXXXXXXX – 3'
BbsI: 5' – **GAAGAC**NNAGGTXXXXXXXXXXXXXXX – 3'
BbvI: 5' – **GCAGC**NNNNNNNNAGGTXXXXXXXXXXXXXXX – 3'
BfuAI: 5' – **ACCTGC**NNNNAGGTXXXXXXXXXXXXXXX – 3'
BsaI: 5' – **GGTCTC**NAGGTXXXXXXXXXXXXXXX – 3'
BsmAI: 5' – **GTCTC**NAGGTXXXXXXXXXXXXXXX – 3'
BsmBI: 5' – **CGTCTC**NAGGTXXXXXXXXXXXXXXX – 3'
BsmFI: 5' – **GGGAC**NNNNNNNNNNAGGTXXXXXXXXXXXXXXX – 3'
BtgZI: 5' – **GCGATG**NNNNNNNNNNAGGTXXXXXXXXXXXXXXX – 3'
FokI: 5' – **GGATG**NNNNNNNNNAGGTXXXXXXXXXXXXXXX – 3'
SfaNI: 5' – **GCATC**NNNNNAGGTXXXXXXXXXXXXXXX – 3'

NOTE: as a general practice it is recommended that two or more additional nucleotides (any sequence) be added to the 5' end of each primer to allow more

efficient cleavage of the PCR product, since some restriction enzymes cleave poorly when its recognition sequence is at the extreme end of a DNA fragment.

Reverse Primer Design

The reverse primer should contain one of the restriction enzyme sites from the multiple cloning site of pP-αSUMO3, to allow directional cloning of your gene of interest into the vector. It is recommended that XbaI be employed as the restriction site in the reverse primer, since the vector will have the BsaI/XbaI-linearized form that can be used directly for ligations without further treatment. An example of a reverse primer for this purpose is:

XbaI: 5' – **TCTAGA**TCAxxx… – 3'

Where **TCTAGA** is the XbaI recognition sequence, TCA is the reverse complement of a stop codon (TGA), and xxx is the reverse complement of the final codon of your gene of interest. Again, it is recommended that extra bases be added to the 5' end, as noted above.

If your insert contains an XbaI site or if the digestion of the PCR insert with a single restriction endonuclease is preferred, restriction site, used in the forward primer could be added in front of XbaI site. For example if BsaI site is added in front of XbaI site, the digestion either with XbaI or BsaI enzyme gives the same 5'CTAG- overhang:

BsmBI/XbaI: 5' – ***CGTCTC*TCTAGA**TCAxxx… – 3'

Any of the polylinker sites could be used for the reverse primer If your gene of interest contains an XbaI site, or if another restriction site is desired for any reason, the other cloning sites available for reverse primer design are SacI, SalI, HindIII, NotI, EagI, and XhoI. Below are examples of reverse primers for each of these sites:

KpnI: 5' – **GGTACC**TCAxxx… – 3'
EcoRI 5' **- GAATTC**TCAxxx… – 3'
BfuI: 5' – ACCTGCNNNNGATCTCAxxx… – 3'
SalI: 5' – **GTCGAC**TCAxxx… – 3'

Note: Only if XbaI site is used in the reverse oligo of the insert, the vector could be digested with a single BsaI restriction endonuclease. If any other polylinker sites are being utilized the vector needs to be digested with a polylinker site enzyme of your choice in addition to BsaI.

Preparation of Insert

The insert should be amplified by PCR to introduce the restriction sites that will generate the appropriate compatible ends as described above. To maintain the sequence integrity of your clone it is sensible to employ a proof reading enzyme such as Pfu (Stratagene), DeepVent (New England Biolabs) or Taq HIFi (Invitrogen) for your PCR reactions. After purification, the PCR product can be digested with the appropriate restriction enzymes in preparation for directly cloning the insert into the pP-αSUMO3 vector. Alternatively, the PCR product can be first cloned into a high copy number plasmid such as pBlueScript (Stratagene) or pCR4.0 TOPO (Invitrogen) generating a clone that can be readily sequenced.

The insert can then be digested out of this plasmid and purified by agarose gel electrophoresis.

Preparation of Vector

The pP-αSUMO3 vector plasmid is provided as a 20µg aliquot of circular vector that has to be digested with BsmBI, or BsmBI in combination with any of the polylinker enzymes, gel-purified and extracted using standard techniques (Sambrook et al.). The digested plasmid can then be used for ligation.

Ligation

For ligation of the prepared insert into the digested pP-αSUMO3 vector, T4 DNA ligase and standard ligation protocols should be employed (Sambrook et al). Since the ligation is directional, phosphatase treatment of vector is unnecessary. The T4 DNA Ligase should be used in the correct buffer and at the appropriate temperature as described by its manufacturer (e.g. MBI Fermentas, New England Biolabs, Roche, Stratagene, Promega).

Transformation

Following incubation of the ligation reactions, plasmids can be transformed into competent *E. coli* by either chemical transformation or electroporation. Because the ligation is directional, there should be little or no occurrence of no-insert background colonies.

Standard bacterial strains like DH5α, TOP10 or others, with the wild type gyrase gene must be used for transformations. These strains show a high propensity for transformation of foreign DNA and have mutations abolishing the activity of the products of the genes RecA and EndA.

Identification of Positives Clones

Using one of the primers used for PCR amplification and an external primer, either the T7 forward or reverse (Sequence) individual transformants can be screened for positive clones. Upon amplification and purification of the plasmid DNA, it should be similarly checked by digestion with a number of restriction enzymes to generate a map.

Pichia Competent Cell Preparation

You will need the following reagents for transforming Pichia and selecting transformants using Zeocin™ as a selection marker. Note: Inclusion of sorbitol in YPD plates stabilizes electroporated cells, as they are somewhat osmotically sensitive.

- 5-10µg pure pP-αSUMO3 vector containing your insert
- YPD Medium
- 50ml conical polypropylene tubes
- 1L cold (+4°C) sterile water (place on ice the day of the experiment)
- 25ml cold (+4°C) sterile 1M sorbitol (place on ice the day of the experiment)
- 30°C incubator
- Electroporation device and 0.2cm cuvettes
- YPDS plates containing 100µg/ml Zeocin™

1. Grow 5ml of your Pichia pastoris strain in YPD in a 50ml conical tube at 30°C overnight.

2. Inoculate 500ml of fresh medium in a 2L flask with 0.1-0.5ml of the overnight culture. Grow overnight again to an OD_{600}=1.3-1.5.

3. Centrifuge the cells at 1500g for 5 minutes at 4°C. Resuspend the pellet with 500ml of ice-cold (0°C), sterile water.

4. Centrifuge the cells as in Step 3, then resuspend the pellet with 250ml of ice-cold, sterile water.

5. Centrifuge the cells as in Step 3, then resuspend the pellet in 20ml of ice-cold, 1M sorbitol.

6. Centrifuge the cells as in Step 3, then resuspend the pellet in 1ml of ice-cold, 1M sorbitol for a final volume of approximately 1.5 ml. Keep the cells on ice and use the same day. Do not store or freeze cells.

Pichia Competent Cell Transformation

1. Mix 80 µl of the cells from Step 6 (above) with 5-10µg of linearized DNA (in 5-10µl sterile water) and transfer them to an ice-cold (0°C) 0.2cm electroporation cuvette. Note: For circular DNA, use 50-100µg.

2. Incubate the cuvette containing the cells on ice for 5 minutes.

3. Pulse the cells according to the manufacturer's instructions for yeast (Saccharomyces
cerevisiae).

4. Immediately add 1ml of ice-cold 1M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15ml tube.

5. Let the tube incubate at 30°C without shaking for 1 to 2 hours.

6. Spread 10, 25, 50, 100, and 200µl each on separate, labeled YPDS plates containing 100µg/ml Zeocin™. Plating at low cell densities favors efficient Zeocin™ selection.

7. Incubate plates from 3-10 days at 30°C until colonies form.

8. Pick 10-20 colonies and purify (streak for single colonies) on fresh YPD or YPDS plates containing 100µg/ml Zeocin™.

Pichia Expression

1. Using a single colony, inoculate 25ml of BMGY in a 250ml baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) until culture reaches an OD_{600} = 2-6 (approximately 16-18 hours). The cells should be in mid-log phase growth.

2. Harvest the cells by centrifuging at 1500-3000g for 5 minutes at room temperature. Decant supernatant and resuspend cell pellet to an OD_{600} = 1.0 in BMMY medium to induce expression (approximately 100-200ml).

3. Place culture in a 1L baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue growth.

4. Add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction.

5. At each of the times indicated below, transfer 1ml of the expression culture to a 1.5ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2-3 minutes at room temperature. Time points (hours): 0, 6, 12, 24 (1 day), 36, 48 (2 days), 60, 72 (3 days), 84, and 96 (4 days).

6. For secreted expression, transfer the supernatant to a separate tube. Store the supernatant and the cell pellets at -80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/alcohol bath. For intracellular expression, decant the supernatant and store just the cell pellets at-80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/alcohol bath.

7. Analyze the supernatants and cell pellets for protein expression by Coomassie stained SDS-PAGE, Western blot, or functional assay

To analyze intracellular protein production wash cell pellet twice with dH_20, resuspend in 200µl of 0.1N NaOH. Keep 10 minutes at room temperature. Pellet the cells and aspirate the NaOH. Resuspend cells in 200µl of SDS-PAGE sample buffer and boil 5 minutes. Centrifuge the suspension and run 1-10µl on an SDS-PAGE gel for protein analysis by staining (e.g. Coomassie blue) or Western blot analysis.

Purification

The presence of a secretory signal followed by a hexahistidine tag at the N-terminus of the hSUMO3 protein sequence allows for simple and rapid purification of fusions by immobilized metal affinity chromatography (IMAC). Follow the nickel resin manufacturer's instructions for use.

Cleavage

Background

SUMO Protease 2, a highly active and robust recombinant protease, cleaves SUMO3 from recombinant fusion proteins. Unlike thrombin, EK, or TEV proteases, whose recognition sequences are short and degenerate, SUMO Protease 2 recognizes the tertiary structure of SUMO3. As a result, SUMO

Protease 2 never cleaves within the fused protein of interest. SUMO Protease 2 cleaves consistently over a broad range of temperature (30°C is optimal), pH [5.5 – 9.5], and ionic strength. SUMO Protease 2 contains a polyhistidine tag at its N-terminus; therefore, SUMO Protease 2 is easily removed from the cleavage reaction by affinity chromatography.

Unit Definition

One unit of SUMO Protease 2 cleaves ~100µg of hSUMO3 Control Protein in 1h at 30°C.

Cleavage

1. Dialyze the purified SUMO fusion proteins for at least 24 h at 4°C against [20 mM Tris-HCl, 150 mM NaCl, pH 8.0, 10% glycerol] or [against PBS]. During the dialysis, change the buffer (~1 L) at least 2 times to effectively remove the detergent and imidazole.
2. Add SUMO Protease 2 at a rate of 1 unit per 100 µg of substrate and incubate at 30°C for 1 h in either Buffer A [20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM dithiothreitol] or Buffer B [PBS pH 7.5, 2 mM dithiothreitol].

Purification

The hSUMO3 fusion tag and SUMO Protease 2 both contain polyhistidine tags at the N-terminus; therefore, remove SUMO-3 and SUMO Protease 2 from the cleavage reaction by affinity chromatography.

Collect protein in flow through. Assess the quality of protein product by examination of a small aliquot on an SDS-PAGE. If the protein is in the appropriate buffer it can be directly used, or further purification steps can be employed.

Controls and Validations

SUMO Protease 2 Control Protein

The protease control protein is a recombinant fusion protein that contains the SUMO3 tag fused to GFP. This fusion protein is used as a positive control for SUMO Protease 2 activity. Uncleaved SUMO3-GFP runs at ~48 kDa while hSUMO3 migrates at 18-20 kDa and GFP runs at ~24 kDa on SDS-PAGE following cleavage

Running a Control with SUMO3-GFP

1. Incubate 100µg SUMO3-GFP and 1 unit of SUMO Protease 2 (total volume100µl) at 30°C for 1h.
2. Remove a 12µL aliquot from 100µl sample and add combine this aliquot with 3µl of 6X SDS-PAGE sample buffer.
3. Heat the sample (15µl) at 95°C for 5 min.
4. Resolve the sample (15 µl), by SDS-PAGE, and stain the gel with Coomassie blue.

Western Blots
Anti- hSUMO3 is an affinity purified, Chicken polyclonal IgY antibody that reacts with SUMO3 in Western blots.

Recommended Dilution(s) for the anti-hSUMO3 Ab.
For immunoblotting, a 1:5,000 dilution is recommended. Most yeast cell lysates can be used as a positive control without induction or stimulation. Researchers should determine optimal dilutions for other applications.

Stock Solutions

10X YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids)
Dissolve 134g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1000 ml of water and filter sterilize. Heat the solution to dissolve YNB completely in water. Store at 4°C. Alternatively, use 34g of YNB without ammonium sulfate and amino acids and 100g of ammonium sulfate. The shelf life of this solution is one year. Note: Pichia cells exhibit optimal growth with higher YNB concentrations; therefore, the amount of YNB used in this kit is twice as concentrated as YNB formulations for Saccharomyces.

500X B (0.02% Biotin)
Dissolve 20mg biotin in 100ml of water and filter sterilize. Store at 4°C. The shelf life of this solution is one year.

10X D (20% Dextrose)
Dissolve 200g of D-glucose in water (1L final volume). Autoclave for 15 minutes or filter sterilize. The shelf life of this solution is one year.

10X M (5% Methanol)
Mix 5ml of methanol with 95ml of water. Filter sterilize and store at 4°C. The shelf life of this solution is two months.

10X GY (10% Glycerol)
Mix 100ml of glycerol with 900ml of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of this solution is one year.

1M potassium phosphate buffer, pH 6.0:
Combine 132ml of 1 M K_2HPO_4, 868 ml of 1M KH_2PO_4 and confirm that the pH = 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH). Sterilize by autoclaving and store at room temperature. The shelf life of this solution is one year.

Media

Low Salt LB (Luria-Bertani) Medium

Low Salt LB medium is needed for use with the Zeocin™ antibiotic. Please note that Low Salt LB can be substituted for regular LB for most applications.

1% Tryptone
0.5% Yeast Extract

0.5% NaCl
pH 7.0

1. For 1L, dissolve the following in 950ml deionized water: 10g tryptone, 5g yeast extract and 5g NaCl
2. Adjust the pH of the solution to 7.5 with NaOH and bring the volume up to 1L.
3. Autoclave for 20 minutes at 15lb/in^2. Let cool to 55°C and add desired antibiotics at this point.
4. Store at room temperature.

Low Salt LB Agar Plates

1. Make Low Salt LB Medium above and add 15g/L agarose before autoclaving.
2. Autoclave for 20 minutes at 15lb/in^2.
3. Let cool to 55°C and add desired antibiotics at this point. Pour into 10cm Petri plates. Let the plates harden, then invert, and store at 4°C.

YPD (1L)

1% yeast extract
2% peptone
2% dextrose (glucose)

1. Dissolve 10g yeast extract and 20g of peptone in 900ml of water. Note: Add 20g of agarose if making YPD slants or plates.
2. Autoclave for 20 minutes on the liquid cycle.
3. Add 100ml of 10X D.

The liquid medium is stored at room temperature. YPD slants or plates are stored at 4°C. The shelf life is 3-4 months.

YPD + Zeocin™ (1L)

1% yeast extract
2% peptone
2% dextrose (glucose)
2% agarose
100µg/ml Zeocin™

1. Dissolve the following in 900 ml of water:
   10g yeast extract
   20g of peptone
2. Include 20g of agarose if making YPD slants or plates.
3. Autoclave for 20 minutes on liquid cycle.
4. Cool solution to 55°C and add 100ml of 10X D. Add 1ml of 100mg/ml Zeocin™, if desired.

Liquid medium without Zeocin™ can be stored at room temperature. Medium containing
Zeocin™ should be stored at 4°C in the dark. YPD slants or plates are stored at 4°C.
The shelf life of medium is 3-4 months. Medium containing Zeocin™ has a shelf life
of 1-2 weeks.

YPDS + Zeocin™ Agar (1 liter)

1% yeast extract
2% peptone

2% dextrose (glucose)
1M sorbitol
2% agarose
100µg/ml Zeocin™

1. Dissolve the following in 900ml of water:
   10g yeast extract
   182.2g sorbitol
   20g peptone
2. Add 20g of agarose.
3. Autoclave for 20 minutes on the liquid cycle.
4. Add 100ml of 10X D
5. Cool solution to ~60°C and add 1.0ml of 100mg/ml Zeocin™.

Store YPDS slants or plates containing Zeocin™ at +4°C in the dark. The shelf life is one to two weeks.

BMGY and BMMY (Buffered Glycerol-complex Medium) and (Buffered Methanol-complex Medium) (1L)

1% yeast extract
2% peptone
100mM potassium phosphate, pH 6.0
1.34% YNB
4 x 10-5% biotin
1% glycerol or 0.5% methanol

1. Dissolve 10g of yeast extract, 20g peptone in 700ml water.
2. Autoclave 20 minutes on liquid cycle.
3. Cool to room temperature, then add the following and mix well:
   100ml 1M potassium phosphate buffer, pH 6.0
   100ml 10X YNB
   2ml 500X B
   100ml 10X GY
4. For BMMY, add 100ml 10X M instead of glycerol.
5. Store media at 4°C. The shelf life of this solution is approximately two months.

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