PROTOPLAST F (Lot 150401)

E-PROTOF **05/15**

Protoplast F is an enzyme cocktail containing fungal cell wall degrading enzymes used to efficiently generate protoplasts of *Aspergillus niger* that are suitable for transformation.

PROPERTIES

1. PRODUCT SPECIFICATION:

Protoplast formation (*A. niger*) ***2 x 10^7 protoplasts/mL**

Protoplast F (Lot 150401) was used to produce protoplasts from wild type *A. niger* N402 with successful subsequent transformation.

**Experimentally determined*

2. ENZYME ACTIVITIES:

***exo*-1,3-β-Glucanase** **495 Units/mL**

One Unit of *exo*-1,3-β-glucanase activity is defined as the amount of enzyme required to release one µmole of glucose reducing sugar equivalents per minute from laminarin (*Laminaria digitata*) (5 mg/mL) in sodium acetate buffer (100 mM) at pH 5.0 and 37°C.

***endo*-1,3-β-Glucanase** **68 Units/mL**

One Unit of *endo*-1,3-β-glucanase activity is defined as the amount of enzyme required to release one µmole of glucose reducing sugar equivalents per minute from 1,3-Beta-Glucazyme HS Tablets (**T-CUR-200T**) in sodium acetate buffer (100 mM) at pH 5.0 and 37°C.

3. STORAGE CONDITIONS:

The enzyme is supplied as an ammonium sulphate suspension and should be stored at 4°C. For assay, this enzyme can be added directly to the protoplasting mixture as outlined in the Protoplast Procedure (section 5). **Swirl to mix the enzyme suspension immediately prior to use.**

4. PREPARATION OF REAGENTS:

A. Malt extract agar (MEA) plates: (4 x 90 mm dia. plates)

4 plates are required per 300 mL of inocullum.

Accurately weigh 5 g of MEA (Sigma cat. no. 70145) and transfer to a 200 mL Duran® bottle containing 100 mL of distilled water. Boil to dissolve and then autoclave at 115°C for 10 min.

Cool to approx. 60°C then aseptically pour approx. 25 mL of the autoclaved solution into a sterile 90 mm petri dish. Leave to cool at room temperature then after the agar is set **store the plates at 4°C and use within 4 days**.

B. Minimal Media (MM): 300 mL

Prepare the individual components as described below and transfer the following amounts of each to a 500 mL Duran® bottle containing approx. 250 mL of distilled water:

3 mL	Ammonium Tartrate Solution (500 mM)*
6 mL	Aspergillus Salt Solution**
0.6 mL	Iron (II) Sulfate (5 mM)***
3 g	Glucose

Stir to dissolve and then adjust the pH to 6.8 using 1 M NaOH. Adjust the final volume to 300 mL with distilled water and autoclave at 105°C for 30 min.

***Ammonium Tartrate Solution (500 mM): 100 mL**

Accurately weigh 9.2 g of ammonium tartrate $(NH_4)_2C_4H_4O_6$ and dissolve in 100 mL distilled water. Autoclave the solution at 105°C for 30 min. Store at room temperature for no longer than 4 weeks.

****Aspergillus Salt Solution: 100 mL**

(350 mM potassium chloride; 105 mM magnesium sulphate; 560 mM potassium phosphate monobasic). Accurately weigh the following components and transfer to a 200 mL Duran® bottle containing 100 mL of distilled water:

2.61 g potassium chloride (KCl)
2.60 g magnesium sulphate ($MgSO_4.7H_2O$)
7.62 g potassium phosphate monobasic (KH_2PO_4)

Stir to dissolve and then autoclave at 105°C for 30 min. Stable for 1 month at 4°C.

*****Iron (II) Sulfate (5 mM): 100 mL**

Accurately weigh 139 mg of $FeSO_4.7H_4O$ and dissolve in 100 mL distilled water. Autoclave the solution at 105°C for 30 min. Stable for 1 month at 4°C.

C. Phosphate Buffered Saline (PBS): 200 mL

Accurately weigh the following components and transfer to a 400 mL Duran® bottle containing 150 mL of distilled water:

1.6 g	sodium chloride (NaCl)
40 mg	potassium chloride (KCl)
288 mg	sodium phosphate dibasic anhydrous (Na_2HPO_4)
48 mg	potassium dihydrogen phosphate (KH_2PO_4)

Stir to dissolve and then adjust the pH to 7.4 using 1 M HCl. Adjust the final volume to 200 mL with distilled water and autoclave at 121°C for 20 min. Do not use if the solution becomes turbid. Stable for 1 month at 4°C.

D. Phosphate Buffered Saline + 0.0005% (v/v) Tween® 80 (PBST): 100 mL

Transfer 100 mL of PBS to a 200 mL Duran® bottle and add 0.1 mL Tween-80 (0.5% v/v). Filter sterilise through a 0.2 µM mixed cellulose ester membrane filter (Sigma cat no. F8148) into a sterile polypropylene tube immediately prior to use. Store at room temperature for up to 4 weeks. Do not use if the solution becomes turbid.

E. Lysis Buffer: 100 mL

(20 mM potassium phosphate; 700 mM potassium chloride)

***Potassium Phosphate Monobasic (25 mM): 100 mL**

Accurately weigh 340 mg of potassium phosphate monobasic (KH_2PO_4), transfer to a 100 mL Duran® bottle containing 100 mL of distilled water and stir to dissolve. Autoclave the solution at 105°C for 30 min. Store at room temperature for no longer than 4 weeks.

***Potassium Phosphate Dibasic (25 mM): 100 mL**

Accurately weigh 435 mg of potassium phosphate dibasic (K_2HPO_4), transfer to a 100 mL Duran® bottle containing 100 mL of distilled water and stir to dissolve. Autoclave the solution at 105°C for 30 min. Store at room temperature for no longer than 4 weeks.

Prepare the lysis buffer fresh on the day of use. Accurately weigh 5.2 g potassium chloride (KCl), transfer to a 100 mL Duran® bottle containing 70 mL of 25 mM potassium phosphate monobasic (KH_2PO_4) and stir to dissolve. Adjust the pH to 5.8 using 25 mM potassium phosphate dibasic (K_2HPO_4) (approx. 10 mL) and bring the volume to 100 mL using distilled water.

5. PROTOPLAST PROCEDURE FOR *ASPERGILLUS NIGER*:

NOTES:
1. Aseptic technique must be used throughout the procedure.
2. The *Aspergillus niger* cultures must be grown on freshly prepared malt extract agar plates that are less than 4 days old.
3. The *Aspergillus niger* cultures must be cultured exactly as described below.
4. Modification of this procedure may adversely affect the efficiency of protoplast formation.

1. Prepare the reagents as described in section 4.
2. Inoculate 4 freshly prepared MEA plates with a sparse amount of *Aspergillus niger* from a plate culture and incubate for 5-7 days at 37°C.
3. Harvest the conidia by pouring approx. 10 mL of sterile PBST onto the surface of each plate and gently release the conidia using a sterile T-shaped spreader.
4. Aseptically pool the contents of each plate into a sterile 50 mL centrifuge tube. Centrifuge the suspension at 4,000 *x g* for 10 min at 4°C and carefully remove the supernatant with a pipette.
5. Resuspend the conidia in 10 mL of sterile ice cold PBS, centrifuge the suspension at 4,000 *x g* for 10 min at 4°C and then carefully remove the supernatant with a pipette.
   Repeat this step once more.
6. Following the final centrifugation step, carefully remove the supernatant with a pipette and resuspend the conidia in sterile ice cold PBS to a concentration of 7 x 10^8 conidia per mL.
   Store at 4°C and use within 3 days of harvest.

NOTE: In order to accurately count the conidia it is necessary to use a haemocytometer according to the manufacturer's instructions.

7. Aseptically transfer 300 mL of minimal media into a sterile 2.5 L straight sided flask, innoculate with 1.5 mL *A. niger* conidia stock, cover the flask with a sterile muslin cloth and incubate in an orbital incubator at 37°C, 200 rpm for 16 h.

NOTE: This incubation should generate a sparse undeveloped mycelium mixture with a harvested weight of ~ 3-4 g/300 mL. 1 g of mycelia is sufficient for each protoplasting experiment. Depending on the needs of the user one reaction is usually sufficient.

8. Following incubation, filter the mycelia through a sterile Miracloth (Merck Millipore cat. no 475855) and remove excess liquid by gently pressing on tissue paper (Do not over dry the mycelia).
9. For each protoplasting reaction accurately measure 9.5 mL of lysis buffer. Swirl the contents of the Protoplast F mixture and add 0.5 mL of this to the lysis buffer. Filter the mixture through a 0.2 µM mixed cellulose ester membrane (Sigma cat no. F8148) into a sterile 50 mL tube (Sarstedt cat no. 62.547.004).
10. Weigh 1 g of mycelium and add to the lysing enzyme mixture and gently pipette using a 10 mL sterile pipette to homogenise the suspension.
11. Secure the reactions horizontally in an orbital incubator and incubate at 30°C, 90 rpm for 30 min. After this time gently homogenise the mixture using a 1 mL sterile pipette tip to ensure a uniform suspension.
12. Continue the incubation and monitor protoplast formation hourly by counting the number of protoplasts using a haemocytometer.

NOTE: A sufficient amount of protoplasts are usually generated after 1 h incubation. Do not incubate for more than 3 h.

13. When an adequate number of protoplasts are observed (normally 1-2 h) the protoplast solution is ready for use. Store at -20°C.