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Product Name & Product No.

Product Name:
Hygromycin B, in DI Water【31282-04-9】
Product No.: H004

Spec.

Chemical Name:
O-6-Amino-6-deoxy-L-glycero-D-galacto-heptopyranosylidene-(1-2-3)-O-beta-D-talopyranosyl(1-5)-2-deoxy-N3-methyl-D-streptamine
Chemical Formula: C20H37N3O13
Molecular Weight: M.W. 527.52
Potency: 1109U/mg
Solubility: Hygromycin is soluble in Water.
Appearance: Solution (100 mg / ml in DI Water), sterile filtered
Purity: >90%

Description

Hygromycin B, an aminoglycosidic antibiotic produced by Streptomyces hygroscopicus, is used for the selection and maintenance of prokaryotic and eukaryotic cells transfected with the hygromycin resistance gene, hph. Hygromycin B kills bacteria, fungi and higher eukaryotic cells by inhibiting protein synthesis. The resistance gene codes for a kinase that inactivates Hygromycin B through phosphorylation. Cloning of the resistance gene and fusion with eukaryotic promoters has resulted in the development of vectors that permit selection for resistance to Hygromycin B in both prokaryotic and eukaryotic cells. Sterile filtered in DI Water.

Working Conc.
Mammalian cells: 50-1000ug/mL; Plant cells: 20-200ug/mL; Bacteria: 20-200ug/mL; Fungi: 200-1000ug/mL

Recommended Procedures

1.1 For the selection of transfected eukaryotic cells

Hygromycin B is added to the culture medium at a concentration that varies with the cell type transfected. A titration experiment for each cell type may therefore be performed to determine the amount of Hygromycin B necessary to kill untransfected cells. A range between 50 ug/ml and 1 mg/ml should be tested.

1. Plate non-transfected cells to be tested at a concentration of 50–200 cells/well in 200 ul culture medium containing various amounts of Hygromycin B (e.g. 50–1000 ug/ml) into microplates (tissue culture grade, 96-wells).
2. Incubate cell cultures for 10–14 days.
3. After 5–7 days replace culture medium by fresh culture medium containing the respective amounts of Hygromycin B if required.
4. Evaluate cellular viability after 10–14 days e. g. with the use of the Cell Proliferation Kit I, the Cell Proliferation Kit II or Cell Proliferation Reagent.

Alternatively 200–500 cells may be plated in 1–2 ml culture medium containing Hygromycin B as described into petri dishes (tissue culture grade, 35 mm) and incubated for 10–14 days. The cytotoxic effect may be determined by evaluation of number of surviving cell colonies or percent confluency.

1.2 Transfection of eukaryotic cells

1.2.1 Preparation of cells for transfection

Adherent cells
Plate the cells one-day before the transfection experiment. The appropriate plating density will depend on the growth rate and the condition of the cells.
Use cells that are 50–80% confluent on the day of the experiment. Plating most cell lines at 1–3 $\times 10^5$ cells in 2 ml in a 35 mm culture dish (or 6-well plate) will achieve this density after overnight incubation.
Suspension cells
Use freshly passaged cells at a concentration between 5 $\times 10^4$ /ml to 1 $\times 10^6$ /ml (2 ml in a 35 mm culture dish or 6-well plate). Determine cell number based on your needs and cell type to be transfected.
1.2.2 Preparation of Transfection Reagent: DNA complex
1. In a small sterile tube, add sufficient serum-free medium as diluent for Transfection Reagent, to a total volume of 100 ul. Add 3 to 6 ul of Transfection Reagent directly into this medium. The order of addition is critical. The serum-free medium must be pipetted into the tube first. Tap gently to mix.
Note: To avoid adversely affecting transfection efficiency, do not allow undiluted Transfection Reagent to come in contact with plastic surfaces other than the pipette tip.
2. Add 1–2 ug DNA solution (0.02–2.0 ug/ul) to the pre-diluted Transfection Reagent from Step 1. Use a total volume of DNA solution between 0.5–50ul.
3. Gently tap the tube to mix the contents. DO NOT VORTEX
Incubate for a minimum of 15 minutes at +15 to +25℃.
Continued incubation for up to 45 minutes(for some cell lines up to 2 hours) will not affect the transfection efficiency in most cell types.
1.2.3 Transfection of cells
1. Dropwise, add the complex mixture from Step 3 (section 1.2.2) to your cells, distributing it around the well. Swirl the wells or flasks to ensure good dispersal.
2. Return the cells to the incubator until the time of the reporter gene assay.
Note: There is no need to remove the reagent: DNA complex from the cells prior to the reporter gene assay. In our experience, exposure of most common laboratory cell types (COS-1, CHO-K1, HEK-293, HeLa) to the reagent: DNA complex until the time of the reporter gene assay (24–48 hours later), has produced no adverse effects, however, this may need to be determined for your particular cell type.

For stable transfection experiments, the complex containing medium can be left unchanged until the cells need to be fed.
3. (optional)
Use serum-free medium during the transfection procedure, and replace with serum-containing medium 3–8 hours after transfection, or add serum directly to wells.

1.3 Selection of transfected cells

Selection of cells, transfected with a DNA construct encoding for hygromycin resistance is performed, using culture medium containing Hygromycin B in a concentration, determined for the particular experimental setup as described above (section 1.1).
1. Remove culture medium and add 5-6 ml fresh culture medium, containing Hygromycin B to a 60 mm culture dish containing the freshly transfected cells. Suspension cells are centrifuged (10 min,

250 ×g) using a sterile centrifugation tube prior to removal of medium and are resuspended in approximately 5 ml culture medium containing Hygromycin B.
2. After 5–7 days replace medium by fresh culture medium containing Hygromycin B as described, if required.
3. Further incubate cells for 5–7 days.
4. After this incubation period the cell cultures will contain only living cells expressing the Hygromycin B resistant phenotype. Therefore culture medium containing Hygromycin B may be replaced by fresh culture medium as described above, but without addition of Hygromycin B.
1.3.1 Subculture of adherent cells
1. After a subcultivation step only viable cells will read here to the culture substrate. This facilitates the evaluation of the cultures and optimizes the culture conditions for the surviving cells.
Note: When trypsin is used, subcultivation may be performed without changing the culture dish by carefully removing the trypsin solution, but not the cells using a fine tipped Pasteur pipette.
2. Resuspend cells in 6 ml culture medium containing Hygromycin B and serum or a trypsin inhibitor.
3. Incubate cells in one and the same culture dish. For suspension cells a dilution of cells may be necessary.
1.3.2 Maintenance of Hygromycin resistant phenotype
For maintenance of the Hygromycin resistant phenotype of established transfected cell lines and for elimination of revertants, cells may be regularity cultured in culture medium containing Hygromycin B at the Sameconcentration used for the initial selection.
Alternatively, the occurrence of revertants may be avoided by permanently culturing the cells in Hygromycin B containing culture medium. In the latter case from the dose response curve as determined above (section 1) a subtonic Hygromycin B concentration may be chosen.

1.4 Cloning of transfected cells

1. Plate cells into a multi well culture plate (tissue culture grade, 96- or 24-wells, according to the particular application) such, that approximately 1 cell will be plated per well in a final volume of 200 ul culture medium for 96-well plates or 1 ml culture medium for 24-well plates, respectively.
2. Incubate cultures according to their respective requirements.
Note: Clones will appear within several days and should be subcultures or passaged according to the particular needs of the cell type, cultured, e.g. when reaching confluency.
3. Prior to dilution and plating steps cell suspensions are to be carefully resuspended
1.4.1 Cloning by picking
1. Plate adherent cells into petri dishes (60 mm or 100 mm, tissue culture grade) at a density of approx. 5×10^3 cells for a 60 mm petri dish or $1– 1.5 \times 10^4$cells for a 100 mm petri dish, respectively.
2. After 5–7 days replace medium by fresh culture medium containing Hygromycin B as described, if required.
3. Further incubate cells for 5–7 days.
4. After this incubation period the cell cultures will contain only living cells expressing the Hygromycin B resistant phenotype. Therefore culture medium

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2. After several days colonies of cells (3–10 cells) will appear.
3. Incubate cultures according to their respective needs.
4. After several days colonies of cells (3–10 cells) will appear.
5. With the aid of an inverted microscope the tip of a fire-polished Pasteur-pipette is placed adjacent to the colony selected and cells from the colony are “picked” by suction.
6. Picked cells are transferred into fresh culture dishes and subsequently cultured according to their respective requirements.

Note: When cloning by picking is to be performed with suspension cells, plating of cells in soft agar is recommended.

FAQS

QUESTION: How can non-transfected cells escape antibiotic selection?

ANSWER: Cells can escape selection if the antibiotic is used at too low concentration or if the cell density on the plate is too high. Additionally, cells rapidly proliferating are killed faster than those proliferating slowly. Control cells should die within 5-7 days after addition of the antibiotic allowing colonies of resistant cells to form by 10-14 days.
QUESTION: How do I determine the Toxic Concentration?
ANSWER: Hygromycin B is added to the culture medium at a concentration that varies with the cell type transfected. A titration experiment for each cell type may therefore be performed to determine the amount of Hygromycin B needed to kill non-transfected cells. The working concentration for mammalian cell selection is normally between 50 ug/ml and 1mg/ml, Plant cells: 20-200 ug/ml, Bacteria: 20-200 ug/ml and Fungi: 200 ug-1mg/ml. Your appropriate concentration should be tested experimentally.
QUESTION: How do I perform a Dose Response curve?
ANSWER: To determine the minimum concentration of antibiotic required to kill your non-transfected host cell line. Test arrange of concentrations (5-6) to ensure that you determine the minimum concentration necessary for your cell line. Seed cells at approxiamately 20-25% confluency on the appropriate number of plates for each time plate and allow cells to adhere overnight. For cells that require higher densities for viability, increase the number of cells seeded. The next day, substitute culture medium with medium containing varying concentration of the antibiotic. Replenish the selective medium every 3-4 days. Count the number of viable cells at regular intervals to determine the appropriate concentration of antibiotic that prevents the growth of non-transfected cells. Select the concentration that kills the majority of the cells in the desired number of days, usually 7-10 days.
QUESTION: How do I maintain Hygromycin resistant phenotype of transfected cell lines?
ANSWER: To maintain Hygromycin resistant phenotype of transfected cell lines and for the elimination of revertants cells may be regularly cultured in culture medium containing Hygromycin B at the same concentration used for the initial selection.
QUESTION: Replacement of Media?
ANSWER: Replacement of culture media containing Hygromycin B is needed only if nutritional components are consumed by the cells cultured. Acidification of the culture medium is a normally a sign of consumption. Utilizing phenol red or media containing phenol red will aid in the detection of acidification. In this case the media will turn yellow.
QUESTION: Is Hygromycin B sensitive to acids?
ANSWER: It is sensitive to high concentrations of acids; however, a brief exposure to dilute acids does not affect its stability.
QUESTION: Can we increase the sensitivity of our cells to Hygromycin B?
ANSWER: The sensitivity to Hygromycin B can be increased by increasing the pH of the medium. Sensitivity appears to be greater at lower salt concentrations.
QUESTION: What is the enzyme that inactivates Hygromycin B?
ANSWER: Hygromycin phosphotransferase (hpt) inactivates the antibiotic hygromycin B through phosphorylation. The hygromycin phosphotransferase gene (denoted hpt, hph or aphIV) codes for hygromycin

phosphotransferase, and is utilized as selectable marker gene for both plant and animal systems.

Storage

Store at 4℃
Handling: Warning! Toxic / Harmful. May be Carcinogenic / Teratogenic. Working hygromycin solutions (<2mg/ml) are stable for ~1 month at 4℃.