B-Hybridoma™ Starting Kit

Higher Colonal Efficiency and Easier Colone Picking

Refined medium suited for B hybridoma growth only

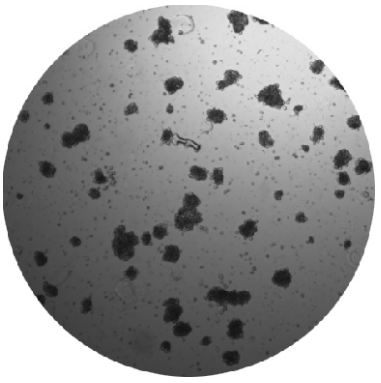
Precise growth factors combination for stronger B cell proliferation

Fine adjusted semisolid medium make picking monocolone easier

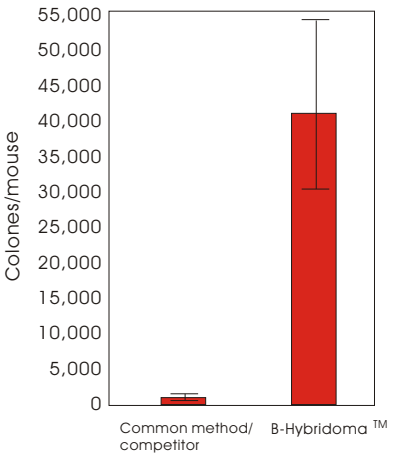
Provided for high throughput monoclonal antibody production

Suited for the use in Genetix automatic picking instrument

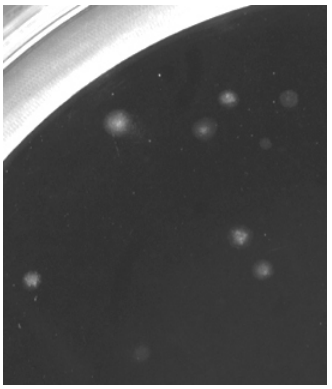
50,000 : 1,000



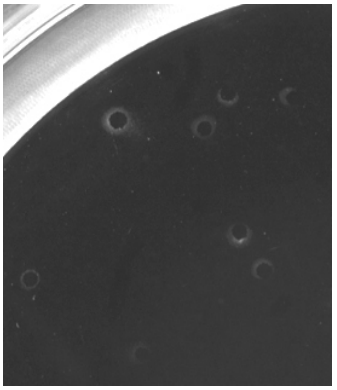
Fused cells culturedin
B-Hybridoma for 7 days



Genetix automatic machine for picking



Before picking



After picking

Table of Contents

1. Introduction

1.1 General Background

Since Dr. Köhler and Milstein described a method to produce monoclonal antibodies (mAbs) in 1975, a variety of modified methods have been developed to increase the efficiency of mAbs production. Most of them are used in some professional laboratories, but are not suitable for common customers. They are time consuming and require many complicated instruments. A problem often encountered in producing mAb is that most hybrid cells are T hybridomas when using whole splenocytes for fusion, these faster growing cells do not synthesize antibodies, resulting in a failure to obtain the desired B hybridomas. A solution to beat this problem is to selectively delete the T hybridomas and pick the B hybridomas as soon as possible after fusion.

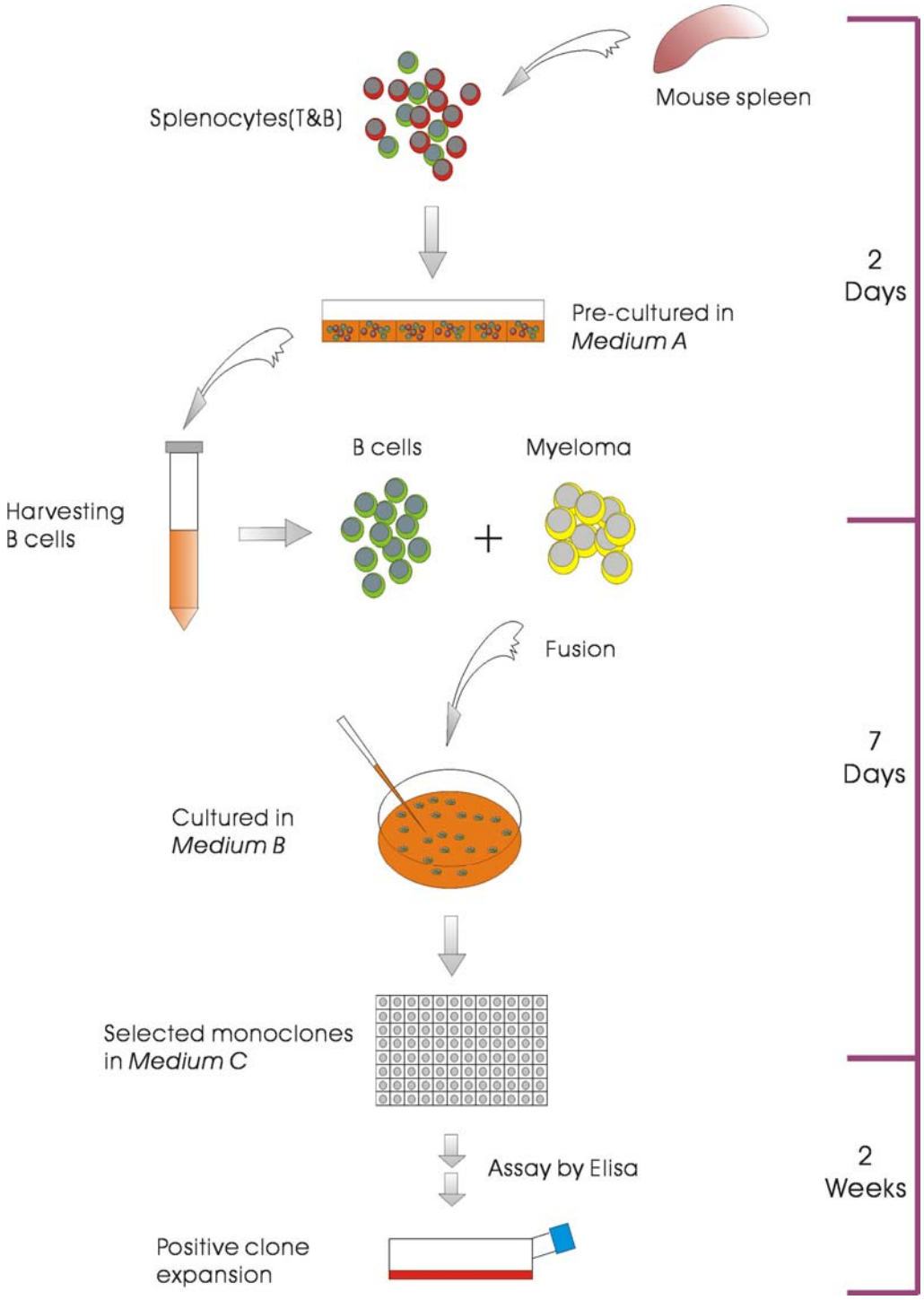
1.2 Description

B-Hybridoma™ starting kit is a special formulation of medium containing B cell growth factors and stimulators, T cell inhibitors and supplements optimized only for the growth of murine B hybridomas. It is a set of mediums, including a pre-culture medium(*Medium A*) supplemented with B cell growth factors and T inhibitors optimized to support the growth of only B cells; semi-solid (*Medium B*) HAT selection medium; and a monoclone expansion medium (*Medium C*) supplemented with B growth factors and pre-selected FCS, optimized for the growth of B hybridomas. First, splenocytes are pre-cultured for 48 hours in *medium A* before fusion, T cell inhibitors in the *medium A* can delete T cells efficiently, avoiding the T hybridoma formation and overgrowth; Then, B cells growth factors in the *medium B* promote B hybridomas growth in the absence of feeder cells. Methycellulose based semisolid medium is convenient for monoclones picking. Combination of optimized PEG for fusion with the medium for B cell growth can produce 20~50-folds-higher clonal efficiency of B hybridomas than common methods. Lastly, HAT selection and cloning of hybridomas are performed simultaneously in a single step, resulting in a substantial saving in time and minimizing the loss of hybridomas due to contamination.
B-Hybridoma™ has made it more efficient to isolate antibody-secreting hybridomas in complex mixtures, thus more suitable for mass production of monoclonal antibodies.

Advantages of B-Hybridoma™ over standard hybridoma selection and cloning methods:

- More than ten thousands of B hybridoma clones can be obtained from 1 spleen after fusion; more hybridomas can be selected and tested.
- Potent B cell growth factors shorten the first cloning time to 5-7days.
- HAT selection and cloning of hybridomas are performed in one step, minimizing both the time and the materials required.
- Direct cloning prevents the overgrowth of potentially valuable slow-growing clones.
- Fewer manipulations are required, therefore the possibility of culture contamination is greatly reduced.
- Growth conditions have been optimized to give high plating efficiency ensuring maximum hybridoma yield.
- B-Hybridoma™ minimizes the screening number of clones.
- Time, cost and labor saving.

B-Hybidoma™ procedure overview



1.3 B-Hybidoma™ products

Medium A (HYSK-B01)	B-HybridomaTM Pre-culture Medium 100 mL Contains RPMI1640, B cell growth factor and stimulators, T cell inhibitors, pre-selected serum, gentamycin, and supplements.
Medium B (HYSK-B02)	B-Hybridoma™ **Selection Medium** 90 mL Contains RPMI1640, B hybridoma growth factors, pre-selected serum, methylcellulose, HAT, gentamycin and other supplements.
Medium C (HYSK-B03)	B-Hybridoma™ **Hybridoma Growth Medium** 500 mL Contains RPMI1640, B hybridoma growth factors, pre-selected serum, HT, gentamycin and supplements.
PEG (HYSK-B04)	B-Hybridoma™ **PEG Solution** 3.2 mL(1.6ml*2) Contains 50% PEG solution pretested for cell fusion in PBS.

1.4 Additional Equipment, Reagents, and Supplies Required

Equipment

Cabinet certified for level II handling of biological materials
Low speed bench centrifuge
37°C incubator with humidity and gas control to maintain >95% humidity and an atmosphere of 5% CO_2 in air
Pipette
Hemacytometer
Inverted microscope
37°C water bath
Liquid nitrogen tank
Freezing container (i.e. Nalgene Catalog #5100)

Reagents

RPMI1640 medium
Fetal Calf Serum
Dimethylsulfoxide (DMSO)
95% Ethanol
Sodium Azide
Trypan Blue

Supplies

50 mL sterile falcon tubes
15 mL sterile falcon tubes
10 mL sterile serological pipettes
5 mL sterile serological pipettes
T-25 cm^2 sterile tissue culture flask
T-75 cm^2 sterile tissue culture flask

96-well sterile tissue culture plates
24-well sterile tissue culture plates
6-well sterile tissue culture plates
96-well ELISA plates
20 mL syringe with16 gauge needle
Forceps
Fine scissors
Fine-mesh disposable cell strainer
Multi-channel pipettor, 8-channel, 20 - 200 µL

Biologicals

Myeloma Cell Line (e.g. SP2/0, x63Ag8.653)
Primed mouse 1 - 4 days after final antigen boost

2. Methods

All procedures should be carried out using sterile technique in a certified biosafety cabinet. All solutions and medium should be prewarmed to 37°C prior to use, unless otherwise stated.

2.1 Mouse Immunization

For immunization procedure, refer to the common method or according to the customer's protocol.

2.2 Splenocytes preparation and preculture

Disaggregate the spleen into a single cell suspension(according to the customer's method), Wash the splenocytes 2 times in 30 mL of RPMI1640, centrifuging at 400 x *g* (~1350 rpm) for 10 minutes each time and removing the supernatant by pipette. After the final wash resuspend the cells with *Medium A* at a concentration of 4 x 10^6/ml and put the cells into 24-well culture plate(1.5ml per well) and cultured for 48 hours in incubator.

2.3 Myeloma Cells and B cells Harvesting

The myeloma cells used for fusion must match the strain of mouse being immunized (e.g. for BALB/c mice the myeloma cells must be of BALB/c origin) and must not secrete any of their own immunoglobulin chains. SP2/0 and X63Ag8.653 are widely used myeloma for fusion.

1. Thaw the parental myeloma cells and culture in RPMI1640 with 10% FCS for at least one week prior to fusion to ensure that the cells are well adapted to culture condition. Seed cells at a density of approximately 1 x 10^5 cells/mL and passage every 2 days. The suggested maximum cell density is approximately 4 x 10^5 cells/mL, although a cell density of up to 8 x 10^5 cells/mL is acceptable.
2. The day before the fusion, split the cells with refresh the medium to ensure the cells are in early-mid log phase growth.

   The total cell mumber for one fusion is about 3 x10^7, the recommended cell density for fusion is 4 x 10^5 cells/mL.

3. Harvest the parental myeloma cells by centrifuging in a 50 mL falcon centrifuge tube at 300 x *g* (~1100 rpm) for 10 minutes. Wash 2 times by adding 30 mL of 37°C pre-warmed serum-free RPMI1640, and repeating the centrifugation. Remove the supernatant by pipette and resuspend the cell pellet in 15 mL of RPMI1640.

   This step should be performed simultaneously with the B cell harvesting to ensure that the myeloma cells are not sitting for an extended period of time.

4. Harvest the precultured splenic cells (cultured for 48 hours) by centrifuging in a 50 ml falcon centrifuge tube at 300 x *g* (~1100 rpm) for 10 minutes. Wash 2 times by adding 30 mL of 37°C pre-warmed serum-free RPMI1640, and repeating the centrifugation. Remove the supernatant by pipette and resuspend the cell pellet in 15 mL of RPMI1640.

5. Count live cells using a viability stain (e.g. Trypan Blue). The viability of parental myeloma cells should be >95%. Calculate the volume of cell suspension that contains 2-3 x 10^7 viable cells. Keep cells at RT or 37°C until fusion.

2.4 Fusion

It is important to remove all the serum adhering to the cells by washing with serum-free RPMI1640*. If the serum is not removed, the PEG will not fuse the cell membranes and the fusion frequency will drop drastically.*

Prepare PEG and mediums (Medium A, B, C) and RPMI1640 for fusion by prewarming to 37°C.

1. Add ~3 x 10^7 parental myeloma cells and ~6 x 10^7 viable B cells (as calculated in Sections 2.3, respectively) to a 50 mL centrifuge tube and centrifuge for 10 minutes at 400 x *g* (~1350 rpm). Aspirate off supernatant. Disrupt the mixed cell pellet by gently tapping the bottom of the tube, the cells will be used for fusion.

   Complete removal of the supernatant is essential to avoid dilution of PEG in the next step.

2. Fuse cells using the method outlined below:

a Slowly add 0.8 mL of B-Hybridoma™ PEG Solution (PEG) to cells suspension, dropwise using a 1 mL pipette, and stir the tube gently at the same time; over a period of 1 minute without stirring; Continually stir the cells gently, with the pipette tip, over the next minute.

   The time of this step must not exceed 3 minutes avoiding the cytotoxicity to the cells

b Add 1 mL prewarmed serum-free RPMI1640 to the fusion mixture slowly in 1 minute, continuously stirring.

c Slowly add 10 mL serum-free RPMI1640 to the cells. Incubate for 10 minutes in water bath at 37°C.

d Slowly add 20 mL of RPMI1640 supplemented with 10% FCS and centrifuge the cells at 200 x *g* (~700 rpm) for 10 minutes. Discard the supernatant and wash cells with 30 mL of serum-free RPMI1640 to ensure that all the PEG is removed.

e Slowly resuspend the cell pellet in 30 mL of RPMI1640 supplemented with 10% FCS and transfer the cell suspension to a T-75 cm^2 tissue culture flask to incubate for 1 hours at 37°C in 5% CO_2

atmosphere.

2.5 Selection and Cloning

Before the day of the fusion, place the selection Medium (*Medium B*) at 2-8°C and thaw overnight. On the day of the fusion, shake vigorously to mix the contents well and let warm to room temperature.

Note: It is not recommended to thaw Medium B in a water bath for heating Medium B above 37°C can cause the methylcellulose to precipitate.

1. Transfer the 10 mL cell suspension(section 2.4 2e) into the 90 mL of *Medium B*. Mix thoroughly by gently inverting the bottle. Let sit for 15 minutes at RT or 37°C to allow the bubbles to rise to the top.
2. Using a 10 mL syringe and 16 gauge blunt-end needle, aseptically plate out 9 mL of cell suspension medium into 1 6-well culture plate(1.5ml per well), totally 11 plates. Tilt the plates to level mixture, and try not to introduce bubbles. Incubate plates at 37°C in 5% CO_2 atmosphere. Do not disturb plates for 7-9 days.

- *Methylcellulose is a viscous solution and cannot be accurately dispensed using pipettes due to adherence of the medium to pipette walls.*
- *Culture conditions are very important to ensure optimal growth of hybridoma clones. We recommend using a water-jacketed incubator. Open and close the incubator door carefully to avoid shaking.*
- *It is important not to disturb especially tilt the plates for the first 4 days. Doing so will result in runny or hazy clones.*

2.6 Harvest, testing and expansion

1. 7-9 days after cells are plated in *Medium B*, examine the plates for the presence of clones visible to the naked eye. A typical fusion will produce more than 10,000 clones over ten plates. Remove isolated clones (usually 500 -1,000 clones are harvested) from the plates using a pipettor set to 10 µL, and sterile pipette tips. Pipette each clone into an individual well of a 96-well tissue culture plate containing 200 µL of *Medium C*. Incubate the plates at 37°C in 5% CO_2 for 3 days without feeding.

- *By the fourth day, each well should have a high cell density and medium that is turning yellow. As the clones have different growth rates, some wells may have media that turns yellow sooner than 4 days.*
- *Use of a stereomicroscope may improve the colony harvesting process.*

2. Transfer 150 µL of supernatant from each hybridoma to a separate well on a new 96-well plate and analyze by using an assay system appropriate for the antigen involved (e.g. ELISA, flow cytometry, Western Blotting, etc.).
3. Add 150 µL of fresh *Medium C* to every well of the original hybridoma containing plates.
4. Gently resuspend the hybridomas that showed a positive response in Step 2. Transfer 100 µL of cells to each of 2 wells of a 24-well plate, containing 1 mL of Medium C.
5. When cells have grown to a suitable density (approximately 4×10^5 cells/mL), freeze the cells from one well and expand the remaining positive clones in a T-25 cm^2 tissue culture flask containing 5 mL of Medium C and 5 mL of FCS supplemented RPMI1640. This step adapts the cells to growth in

customer's modified culture medium. In addition, keep a sample of cells in Medium C, in case the cells don't adapt well to the mixed medium.

6. When cells have grown to a suitable density (approximately 4 x 10^5 cells/mL), transfer 5 mL of cell culture by pipette into 30 mL of commonly used RPMI1640 complete medium in a T-75 cm^2. More aliquots of cells can be frozen at this point in order to secure the supply of the hybridoma.

3. Troubleshooting

Listed below are some of the most common problems associated with the generation, selection and cloning of hybridomas for the purpose of producing specific monoclonal antibodies.

3.1 Low Number of Hybridomas After Fusion and Selection

Typically a good fusion should yield 10,000 to 30,000 clones in 10 plates. A considerably lower number of hybridomas may be the result of a low fusion rate or a low viability and cloning efficiency of the hybridomas.

Possible causes for a low fusion rate:

- Serum was not efficiently removed from the cells prior to the fusion (Sections 2.2 and 2.3). Any protein still present when PEG is added will greatly reduce the fusion efficiency.
- PEG concentration was too low due to incomplete removal of the supernatant after centrifugation of the spleen cell/myeloma cell mixture prior to the addition of PEG (Section 2.4).
- The cell pellet was not sufficiently disrupted prior to the addition of PEG.
- The cells were exposed to PEG for too long resulting in cell death.

Possible causes for a low hybridoma viability and cloning efficiency:

- Poor growth or low viability of myeloma cells prior to fusion. Poor growth may occur if myeloma cells have not been sufficiently adapted to RPMI1640 if the cells comonly cultured in DMEM or after initiating the culture from cryopreserved cells. Low myeloma viability may also occur if myeloma cell density is too high on the day of the fusion.
- Myeloma cells are contaminated with *mycoplasma*. *Mycoplasma* consume thymidine, which can result in low numbers of recovered hybridomas during the HAT selection.
- Poor viability of spleen cells prior to fusion. Poor viability may be due to age and health status of the immunized mouse, the cells overgrowth in the preculture step or an extended period of time between B cells harvesting and fusion. It is recommended to work quickly and perform the fusion as soon as possible (preferably within 1 hour) after harvesting the cells.
- Poor viability of the fused cells. Freshly fused cells are very fragile and should treated gently between fusion and plating. Rapid changes in temperature and vigorous pipetting should be avoided as this may result in rupture of the plasma membrane and cell death.

3.2 Too many hybridomas after fusion

- Normally one third of fused cells were used for culture with 100 mL *Medium B* in 11 6-well plates, it will yield 10,000-30,000 B hybridoma clones, and it is enough for picking and screening.
- Do not put all fused cells into 90 mL *Medium B*, otherwise too many clones will exhaust the nutrition before the clones can be seen by naked eyes.

3.3 No or Too Few Positive Hybridomas

Assuming that the total number of hybridomas generated was normal, a lack of positive hybridomas may have several causes, including:

- Too low dose or low immunogenicity of the antigen. The optimal dose and immunogenicity is

dependent on the type of antigen used and can only be determined empirically. Typically 20 - 100 µg of purified antigen works well, but much lower doses (nanograms) have also been used successfully.

- Sub-optimal immunization schedule, resulting in too few specific antibody-forming cells at the time of fusion. The most optimal immunization schedule is dependent on the type and dose of antigens and desired affinity of the specific antibodies. As a general principle, the longer the time interval between injections, the higher the affinity of the antibodies produced.

3.4 Hazy or Runny Clones

- Methylcellulose is a viscous solution. Disturbing the dishes before Day 6 will break apart small forming clones and cause them to appear hazy or runny.

3.5 Other

- If clones in Medium B are not well distributed due to low viscosity of the medium, or if the clones are hazy or runny, or if the cell density is too high, plucking individual clones might be difficult. In this case we recommend recloning positive hybridomas once the cells have been established in RPMI1640.
- It will be not easier to select too many clones in one well due to the higher clonal efficiency of the system. So the fused cells should be diluted several folds according to the customer's experience.

4. References

1. Köhler G, Milstein C: Continuous culture of fused cells secreting antibody of predefined specificity. Nature 256: 495-497, 1975.
2. Harlow E, Lane D: Monoclonal Antibodies, Chapter 6 in: Antibodies, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York: ISBN 0-87969-314-2, 1988.
3. Davis JM, Pennington JE, Kubler A-M, Conscience JF: A simple, single-step technique for selecting and cloning hybridomas for the production of monoclonal antibodies. J Immunol Methods 50: 161-171, 1982.
4. Goding JW: Antibody production by hybridomas. [Review]. J Immunol Methods 39: 285-308, 1980.
5. Kennett RH, McKearn TJ, Bechtol KB: Monoclonal antibodies. Plenum Press, New York, 1980.
6. Harlow Ed, Lane David. Using antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York: ISBN 0-87969-543-9, 1999.
7. Coligan JE: Current Protocols in Immunology, Unit 2.5: Production of Monoclonal Antibodies. John Wiley & Sons, Inc., USA, 2006.