TECHNICAL MANUAL

ClonaCell™-HY: A Complete Workflow for Hybridoma Generation

STEMCELL™ TECHNOLOGIES

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1.0 Introduction

1.1 Background

Hybridoma technology has been widely used for antibody discovery since George Köhler and César Milstein first described a method to produce monoclonal antibodies (mAbs) of defined specificity in 1975. They were later awarded the Nobel Prize in Physiology and Medicine[1] for their discovery. Hybridoma generation is a critical upstream step on the path towards monoclonal antibody discovery for diagnostic applications, biomedicine or biologics production, or discovery research. Developing hybridomas involves many complex steps that begin with injecting an animal (e.g. mouse or rat) with the desired antigen to generate a robust immune response. Primary B cells harvested from the spleen or lymph nodes are capable of producing antibodies but have a limited lifespan in culture. The key discovery of Köhler and Milstein was to fuse these B cells with myeloma cells to generate hybridomas that are capable of growing indefinitely in culture. A small portion of the hybridomas generated by this fusion process secretes antibodies specific for the chosen antigen. By careful selection, screening, and subcloning, it is possible to isolate stable hybridoma cell lines that constitutively secrete monoclonal antibodies of appropriate specificity.

A variety of methods have been used to fuse, grow, select, and clone hybridomas.[2-6] A requirement for the production of monoclonal antibodies for therapeutic applications is that the expressing hybridoma cell line is clonal, that is, derived from a single cell. However, in practice, an entirely homogeneous population is unrealistic; many industrially important hybridoma cell lines show deviations in growth rate and expression of antibodies despite repeated rounds of subcloning.[7-9] In addition, low expressers have a tendency for an increased growth rate due to less energy being diverted to antibody production and more to growth, thus making up the majority of the population.[8,10] Slower-growing expressing clones may be lost due to overgrowth of faster-growing non-expressing clones.

Conventionally, the limiting dilution cloning technique for screening for high antibody-producing clones is used. This technique involves dispensing a mixed population of cells into 96-well plates at an average of less than one cell per well. Wells that are observed to contain single cells are selected for ELISA analysis after expansion, and several rounds of subcloning may be required to reach an acceptable probability of monoclonality. Limiting dilution is widely accepted, but it can be time-consuming and labor-intensive.

Another method for hybridoma selection and cloning uses a methylcellulose-based semi-solid medium. By plating the cells in a semi-solid selection medium after fusion, the progeny of the selected cells remain localized together during selection and form distinct colonies that can be harvested and screened individually. Each distinct colony is derived from a single cell, thus, colonies formed in semi-solid medium have a high likelihood of being clonal. Slower-growing clones are physically separated from faster-growing clones by the semi-solid matrix, preventing overgrowth (as can occur during selection in liquid media) and allowing for easier isolation and screening. The ClonaCell™-HY semi-solid method eliminates the possibility of losing potentially valuable and rare clones due to overgrowth and increases the diversity of clones that can be identified and isolated.[11] Hybridoma selection and cloning are performed in a single step, resulting in substantial time savings in generating stable clonal cell lines.

STEMCELL Technologies' ClonaCell™-HY product line offers specialized media containing growth factors and supplements optimized for all stages of hybridoma development, including selection with hypoxanthine, aminopterin, thymidine (HAT), as well as cloning and expansion.

1.2 Description

The ClonaCell™-HY line of media offers a complete solution for hybridoma generation and selection, regardless of whether your preferred workflow incorporates selection in a liquid or semi-solid medium.

Advantages of the ClonaCell™-HY system include:

Versatility

- Compatible with conventional limiting dilution cloning and semi-solid cloning methods.
- All the necessary media and reagents are available for every step of the hybridoma development workflow, from cell fusion to expansion of selected hybridomas.
- Verified for use in mouse and rat hybridoma development. Reportedly compatible with human and hamster hybridoma development.
- Compatible with polyethylene glycol (PEG)-based fusion and electrofusion.
- Hybridomas can be transferred without an adaptation step from serum-containing medium used during selection to a serum-free, animal origin-free (AOF) medium for liquid cloning and/or expansion.
- Serum-free (AOF) liquid medium facilitates detection and purification of the desired hybridoma-derived antibodies without interference from contaminating serum-derived immunoglobulins.

Productivity

- Growth conditions have been optimized to give high plating efficiency, ensuring maximum hybridoma yield.
- Direct cloning in semi-solid medium prevents the overgrowth of potentially valuable slow-growing clones.
- Hybridoma colonies have a high probability of being monoclonal from the start, so subcloning may not be necessary.

Reliability

- ClonaCell™-HY products are manufactured using pre-screened components to ensure lot-to-lot consistency, saving time on screening and troubleshooting medium components.
- The antibiotic gentamicin is included in the media to suppress growth of a broad range of potential microbial contaminants such as mycoplasma.

1.3 ClonaCell™-HY Products

PRODUCT	CATALOG #	SIZE	DESCRIPTION	CONTAINS
ClonaCell™-HY Hybridoma Kit	03800	1 Kit	Media and reagents for all steps in hybridoma development and monoclonal antibody production	• ClonaCell™-HY Medium A • ClonaCell™-HY Medium B • ClonaCell™-HY Medium C • ClonaCell™-HY Medium D • ClonaCell™-HY Medium E • ClonaCell™-HY PEG
ClonaCell™-HY Medium A	03801	500 mL	Myeloma and hybridoma culture medium	Dulbecco's Modified Eagle's Medium (DMEM), pre-selected serum, gentamicin, 2-mercaptoethanol, phenol red, L-glutamine, other supplements and ingredients
ClonaCell™-HY Medium B	03802	500 mL	Hybridoma fusion medium used to wash cells prior to cell fusion and for use during fusion	DMEM, gentamicin, 2-mercaptoethanol, phenol red, L-glutamine, other supplements and ingredients
ClonaCell™-HY Medium C	03803	100 mL	Fusion recovery medium to promote hybridoma viability after fusion prior to HAT selection	DMEM, pre-selected serum, gentamicin, 2-mercaptoethanol, phenol red, L-glutamine, other supplements and ingredients
ClonaCell™-HY Medium D	03804	90 mL	Semi-solid methylcellulose-based hybridoma selection and cloning medium with HAT (serum-containing)	DMEM, methylcellulose, pre-selected serum, HAT, gentamicin, 2-mercaptoethanol, phenol red, L-glutamine, other supplements and ingredients
ClonaCell™-HY Medium E	03805	500 mL	Hybridoma growth medium to support hybridoma growth and expansion after HAT selection.	DMEM, pre-selected serum, HT, gentamicin, 2-mercaptoethanol, phenol red, L-glutamine, other supplements and ingredients
ClonaCell™-HY PEG	03806	1.5 mL	Pre-tested reagent for hybridoma fusion	50% (w/v) solution of polyethylene glycol in DMEM, gentamicin, phenol red
ClonaCell™-HY Medium D without HAT	03810	90 mL	Semi-solid methylcellulose-based hybridoma selection and cloning medium, without HAT (serum-containing)	DMEM, methylcellulose, pre-selected serum, gentamicin, 2-mercaptoethanol, phenol red, L-glutamine, other supplements and ingredients
ClonaCell™-HY Liquid HAT Selection Medium	03831	500 mL	Hybridoma medium containing HAT for selection and cloning in liquid suspension cultures	DMEM, pre-selected serum, HAT, gentamicin, 2-mercaptoethanol, phenol red, L-glutamine, other supplements and ingredients
ClonaCell™-HY AOF Expansion & Cloning Medium	03835	500 mL	Animal origin-free liquid medium to support hybridoma expansion and subcloning after HAT selection	Basal medium, HT, gentamicin, phenol red, L-glutamine, recombinant proteins, other supplements and ingredients

2.0 Materials, Reagents, and Equipment

2.1 Materials

- Sterile conical tubes: 15 mL (Catalog #38009) and 50 mL (Catalog #38010)
- Sterile serological pipettes:
  - 1 mL (Catalog #38001)
  - 2 mL (Catalog #38002)
  - 5 mL (Catalog #38003)
  - 10 mL (Catalog #38004)
  - 25 mL (Catalog #38005)
- T-25 cm^2 and T-75 cm^2 sterile tissue culture flasks
- Sterile tissue culture-treated flat-bottom plates:
  - 96-well (Catalog #38022)
  - 24-well (Catalog #38017)
  - 6-well (Catalog #38015)
- 96-well round-bottom microplate (Catalog #38018)
- 100 mm Petri Dish (Catalog #27110)
- 12 mL syringe
- Blunt-End Needles, 16 Gauge (Catalog #28110)
- Forceps
- Fine scissors
- Cell Strainer (e.g. Catalog #27305 or 27260 or 27250)
- Multi-channel pipettor, 8-channel or 12-channel, 20 - 200 µL (e.g. Catalog #38064)
- Plastic container with lid
- Cryogenic vials (e.g. Catalog #38047)

2.2 Reagents

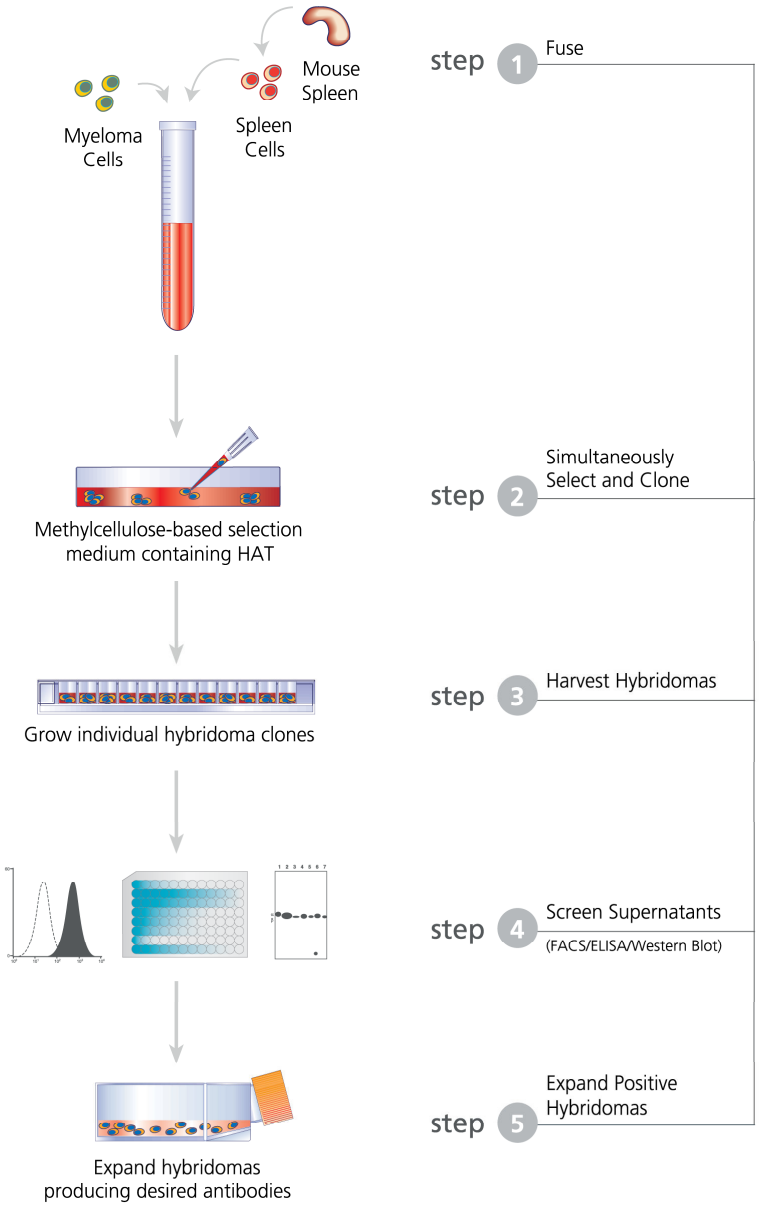
- Sterile distilled water
- 3% Acetic Acid with Methylene Blue (Catalog #07060)
- Trypan Blue (Catalog #07050)
- Dimethylsulfoxide (DMSO)
- 95% ethanol
- Sodium azide

2.3 Equipment

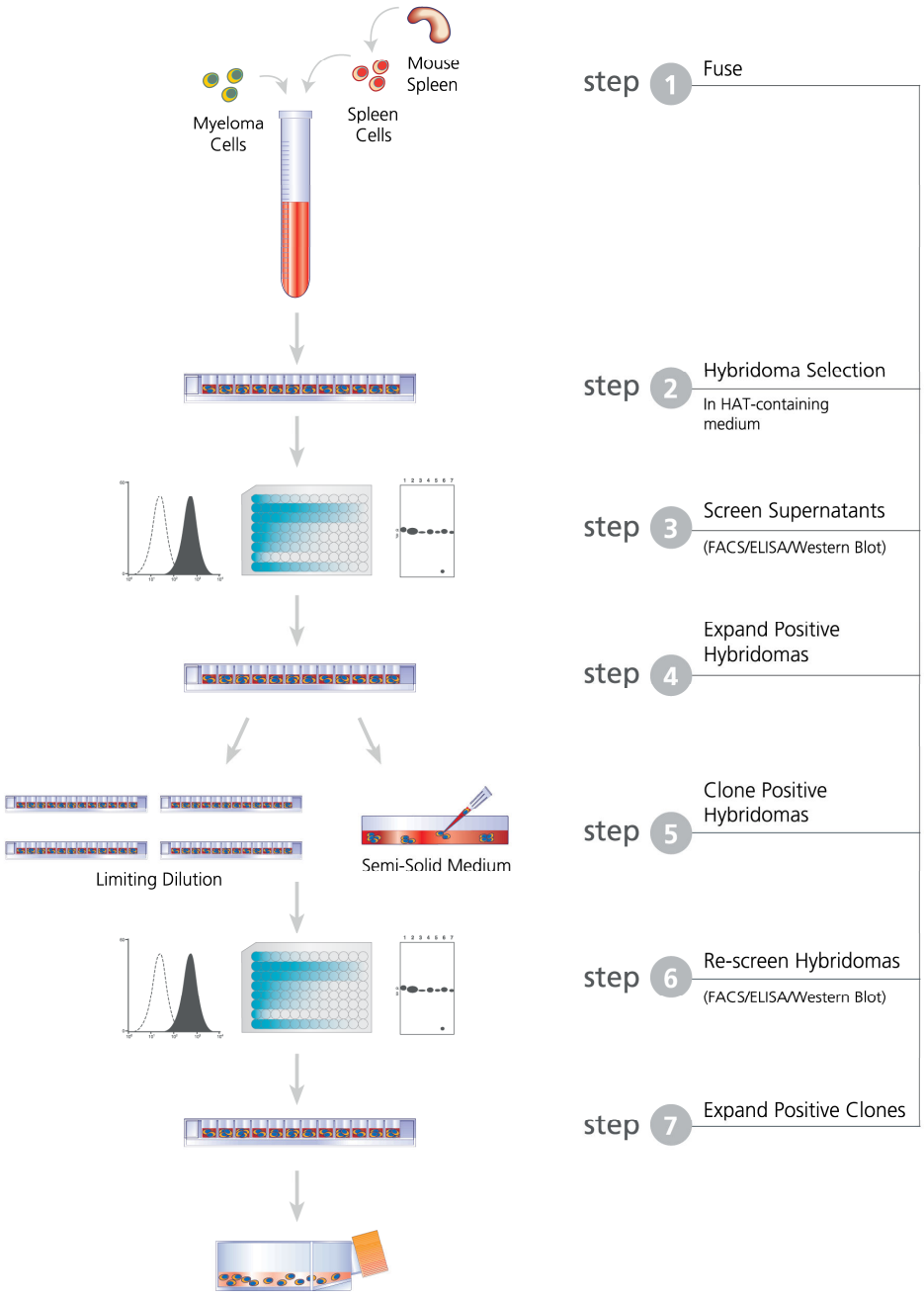
- Biohazard safety cabinet certified for Level II handling of biological materials
- Low-speed bench centrifuge
- Incubator at 37°C with 5% CO_2 in air and ≥ 95% humidity
- Pipette-aid
- Hemocytometer
- Routine light microscope
- Inverted microscope
- 37°C water bath
- Liquid nitrogen tank and freezing head
- Optional: Freezing container (e.g. Nalgene Catalog #5100)

3.0 Protocol Diagrams

3.1 Selection and Cloning Using Semi-Solid Medium



3.2 Selection and Cloning Using Liquid Medium



4.0 Methods

Perform all protocols using sterile technique in a certified biosafety cabinet. Warm all media and reagents to 37°C prior to use, unless otherwise stated.

For a suggested mouse immunization schedule, see Appendix I.

4.1 Preparation of Myeloma Cells

The parental myeloma cells must not secrete any of their own immunoglobulin chains and should not have a functional HGPRT. They should fuse well and allow the formation of stable hybridomas that will continue to secrete specific monoclonal antibodies. Cells should also be mycoplasma-free. Parental myeloma cells that meet these criteria (such as Sp2/0-Ag14 and X63-Ag8.653) are widely available. Be sure to obtain a parental myeloma cell that has been proven to yield robust, stable hybridomas.

1. Thaw the parental myeloma cells (see Appendix IV) and culture in either ClonaCell™-HY Medium A or ClonaCell™-HY AOF Expansion & Cloning Medium for at least 1 week prior to fusion to ensure that the cells are well adapted to the medium. Seed cells at a density of approximately 5 x 10^4 cells/mL and passage every 2 days. The suggested maximum cell density is 4 x 10^5 cells/mL, although a cell density of up to 8 x 10^5 cells/mL is acceptable.

   Note: If cells are allowed to grow beyond 8 x 10^5 cells/mL, passage at least 2 times to bring them back to early-mid log phase growth prior to fusion.

2. Test parental myeloma cells for mycoplasma prior to fusion. If myeloma cells are positive for the presence of mycoplasma, do not proceed with fusion; thaw a new vial or obtain new myeloma cells.

3. Calculate the cell growth rate at every passage. The day before the fusion, count the viable cells and split so that at least 2 x 10^7 parental myeloma cells will be available the next day.

   Note: The recommended cell density for fusion is 2 x 10^5 cells/mL. Only 100 mL of cells is needed, but 200 mL should be cultured to ensure sufficient cell numbers for fusion.

4. Harvest the parental myeloma cells as follows:

   i. Centrifuge the parental myeloma cells in a 50 mL conical tube at 300 x *g* for 10 minutes at room temperature (15 - 25°C) or 37°C. Remove and discard the supernatant.
   ii. Add 30 mL of ClonaCell™-HY Medium B and centrifuge at 300 x *g* for 10 minutes at room temperature (15 - 25°C) or 37°C. Remove and discard the supernatant. Repeat twice for a total of 3 washes.
   iii. Resuspend the cell pellet in 25 mL of Medium B.

   Note: Harvesting/washing may be performed simultaneously with, or after, the spleen cell preparation (section 4.2) to ensure that the myeloma cells are not sitting for an extended period of time. It is important to remove all the serum adhering to the cells by washing with serum-free Medium B. If the serum is not removed, the PEG will not fuse the cell membranes and the fusion frequency will drop drastically. If cells were cultured in ClonaCell™-HY AOF Expansion & Cloning Medium, washing with Medium B is still recommended to prepare cells for fusion.

5. Count live cells using a viability dye (e.g. Trypan Blue). The viability of the parental myeloma cells should be > 95%.

6. Calculate the volume of cell suspension that contains 2 x 10^7 viable cells. Keep cells at room temperature (15 - 25°C) or 37°C until fusion (section 4.3).

4.2 Preparation of Splenocytes

1. Disaggregate the spleen into a single-cell suspension as described in Appendix II.
2. Wash the splenocytes as follows:
   i. Add 30 mL of ClonaCell™-HY Medium B.
   ii. Centrifuge at 400 x *g* for 10 minutes at room temperature (15 - 25°C) or 37°C. Remove the supernatant using a pipette and discard.
   iii. Repeat steps i and ii twice, for a total of 3 washes.
   iv. After the final wash, resuspend the cells in 25 mL of Medium B.

   Note: It is important to remove all the serum adhering to the cells by washing with serum-free Medium B. If the serum is not removed, the PEG will not fuse the cell membranes and the fusion frequency will drop drastically.
3. Prepare a 1 in 10 dilution of cells in 3% Acetic Acid with Methylene Blue.

   For example, mix 10 µL of the cell suspension with 90 µL of 3% Acetic Acid with Methylene Blue.
4. Count cells in this diluted sample using a hemocytometer. Calculate the volume of cell suspension that contains 1 x 10^8 cells.
5. Place cells at room temperature (15 - 25°C) or 37°C until fusion (section 4.3).

   Note: Splenocytes may be enriched for antibody-expressing cell types such as B cells or plasma cells prior to performing fusions using one of the following EasySep™ cell separation kits:

   - EasySep™ Mouse B Cell Isolation Kit (Catalog #19854)
   - EasySep™ Mouse Pan-B Cell Isolation Kit (Catalog #19844)
   - EasySep™ Mouse CD19 Positive Selection Kit II (Catalog #18954)

4.3 Hybridoma Fusion

1. Choose a selection medium for the day after fusion (section 4.4):
   - Semi-solid selection: ClonaCell™-HY Medium D
   - Liquid selection: ClonaCell™-HY Liquid HAT Selection Medium

   Place the medium at 2 - 8°C to thaw overnight.
2. Proceed with either electrofusion (section 4.3.1) or PEG-mediated fusion (section 4.3.2).

4.3.1 Electrofusion

ClonaCell™-HY media have been tested and confirmed to be compatible with multiple electrofusion methods and instruments for hybridoma generation. Refer to manufacturer's recommended fusion conditions for the electrofusion instrument used.

4.3.2 Polyethylene Glycol (PEG)-Mediated Fusion

1. Warm ClonaCell™-HY PEG, Medium A, Medium B, and Medium C to 37°C.
2. Prepare a 37°C water bath (for step 8).
3. Add 2 x 10^7 parental myeloma cells (volume determined in section 4.1) and 1 x 10^8 viable splenocytes (volume determined in section 4.2) to a 50 mL conical tube.

4. Centrifuge at 400 x *g* for 10 minutes. Remove the supernatant using a pipette.

   Note: Complete removal of the supernatant is essential to avoid dilution of PEG in step 6.

5. Disrupt the cell pellet by gently tapping the bottom of the tube.
6. Slowly add 1 mL of ClonaCell™-HY PEG to the cell pellet dropwise using a 1 mL serological pipette over a period of 1 minute without stirring. Following this, continuously stir the cells gently with the pipette tip for 1 minute.
7. Add 4 mL of Medium B to the fusion mixture, continuously stirring for 4 minutes.
8. Slowly add 10 mL of Medium B to the fusion mixture. Incubate in a 37°C water bath for 15 minutes.
9. Slowly add 30 mL of Medium A. Centrifuge at 400 x *g* for 7 minutes. Remove and discard the supernatant using a pipette, being careful not to disturb the cell pellet.
10. Slowly add 40 mL of Medium A to resuspend the cell pellet. Centrifuge at 400 x *g* for 7 minutes. Remove and discard the supernatant using a pipette, being careful not to disturb the cell pellet.

    Note: This wash step is necessary to ensure that all PEG is removed.

11. Slowly resuspend the cell pellet in 10 mL of Medium C.
12. Transfer the cell suspension to a T-75 cm^2 tissue culture flask containing 20 mL of Medium C (total culture volume = 30 mL).
13. Incubate the flask at 37°C and 5% CO_2 for 16 - 24 hours.
14. Proceed to section 4.4 for selection and cloning.

4.4 Hybridoma Selection

Hybridoma selection and cloning may be performed simultaneously using methylcellulose-based semi-solid medium such as ClonaCell™-HY Medium D (section 4.4.1). For selection in liquid medium, ClonaCell™-HY Liquid HAT Selection Medium is recommended (section 4.4.2).

4.4.1 Selection and Cloning in Semi-Solid Medium

The following protocol is for hybridoma selection and cloning in ClonaCell™-HY Medium D in 100 mm Petri dishes. For an alternative protocol using 96-well plates, see Appendix III.

1. On the day after fusion, shake ClonaCell™-HY Medium D vigorously to mix thoroughly and warm to room temperature (15 - 25°C).
2. Transfer fused cell suspension from the T-75 cm^2 flask into a 50 mL conical tube and centrifuge at 400 x *g* for 10 minutes at room temperature (15 - 25°C) or 37°C. Remove the supernatant using a pipette.
3. Resuspend the cells in ClonaCell™-HY Medium C to a total volume of 10 mL.

   Note: It is critical to not exceed the 10 mL final volume. If you wish to add any additional cytokines or growth factors to Medium D, include this volume in the total 10 mL resuspension volume.

   Note: To achieve optimal colony density for colony picking, plating at several cell densities is recommended, as fusion efficiency may vary.

4. Transfer the cell suspension (10 mL) directly into the bottle containing 90 mL of Medium D. Mix thoroughly by gently inverting the bottle several times. Incubate at room temperature (15 - 25°C) or 37°C for 15 minutes to allow bubbles to rise to the top.

5. Using a 12 mL syringe with a 16-gauge blunt-end needle, slowly add 9.5 mL of the cell suspension in Medium D to each of 10 x 100 mm Petri dishes, being careful to avoid bubbles during plating. Tilt each plate to evenly distribute the medium across the bottom of the plate.

   Note: Do not use pipettes to dispense methylcellulose, as the volume dispensed will not be accurate. Syringes and large bore blunt-end needles should be used for accurate dispensing of viscous methylcellulose medium and to prevent needle-stick injuries.

6. Incubate plates at 37°C and 5% CO_2 for 10 - 14 days. Do not disturb plates.

   Note: Culture conditions are important to ensure optimal growth of hybridoma colonies. We recommend using a water-jacketed incubator. Place the plates in a lidded plastic container with a layer of sterile distilled water to maintain moisture content. Open and close the incubator door carefully to avoid shaking. It is important not to disturb the plates for the first 10 days; doing so will result in runny or diffuse colonies.

7. Proceed to section 4.5.1 for harvesting colonies.

4.4.2 Selection in Liquid Medium

1. On the day after fusion, invert the bottle of ClonaCell™-HY Liquid Hat Selection Medium several times to mix well. Warm to 37°C before use.

   Note: If not used immediately, store at 2 - 8°C for up to 2 weeks. Alternatively, aliquot and store at -20°C. Do not exceed the expiry date as indicated on the label.

2. Transfer fused cell suspension from the T-75 cm^2 flask into a 50 mL conical tube and centrifuge at 400 x *g* for 10 minutes at room temperature (15 - 25°C) or 37°C. Remove the supernatant using a pipette.
3. Resuspend the cells in ClonaCell™-HY Liquid HAT Selection Medium to a total volume of 20 mL.
4. Transfer the 20 mL cell suspension into 180 mL of ClonaCell™-HY Liquid HAT Selection Medium. Mix thoroughly by gentle inversion.
5. Using a multi-channel pipettor, aseptically add 200 µL of cell suspension into each well of 10 x 96-well tissue culture plates.
6. Incubate plates in a humidified incubator at 37°C and 5% CO_2 for 10 - 14 days.
7. Proceed to section 4.5.2 for harvesting colonies.

   Note: Cells are not monoclonal at this stage.

4.5 Harvesting Hybridomas

Hybridomas are typically ready to harvest after 14 days. A typical fusion will produce 1000 or more colonies across the 10 plates. Please refer to the appropriate sections below, based on the method used in the selection step.

4.5.1 Harvesting From Semi-Solid Medium

1. Examine the plates for the presence of colonies visible to the naked eye.
2. Remove isolated colonies (usually 500 - 1000 colonies 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 ClonaCell™-HY Medium E or ClonaCell™-HY AOF Expansion & Cloning Medium.

   Note: Fully automated colony picking can be performed with an appropriate instrument instead of manual picking.

   Note: Pick clones of different sizes, as slower-growing clones (i.e. smaller colonies) are often very good antibody producers. Such slow-growing hybridomas are often missed in other hybridoma screening procedures. Use of a stereomicroscope may improve the colony harvesting process. Placing a mirror under the dish may facilitate harvesting of colonies by allowing for easier alignment of the pipette tip with the colony.
3. With the pipettor set to 150 µL, pipette the entire contents of each well up and down several times to resuspend the colony.

   Note: The colony does not need to be resuspended into an entirely homogeneous single-cell suspension but should be dispersed sufficiently to achieve good growth. Resuspension of the colonies may be performed using a multi-channel pipettor after all selected colonies have been transferred to the 96-well plate. Ensure a new sterile tip is used for each clone to maintain clonality of the colony.
4. Incubate plates at 37°C and 5% CO_2 for 3 - 4 days prior to screening (section 4.6).

   Note: After 3 - 4 days, each well should have a high cell density and medium that is turning yellow. As the colonies have different growth rates, some wells may have medium that turns yellow earlier.

4.5.2 Harvesting From Liquid Medium

This is an optional step in which the cells are passaged once before screening, to reduce the rate of false positives in preliminary screen due to carryover IgG from parental B cells that have died off during selection.

1. Using a multi-channel pipettor, passage fusion products by aseptically transferring 50 µL of cell suspension from each 96-well plate to a new 96-well plate with 150 - 200 µL of fresh Liquid HAT Selection Medium in each well.
2. Incubate at 37°C and 5% CO_2 for 3 - 4 days prior to screening (section 4.6).

4.6 Screening and Expanding Hybridomas

1. Transfer 50 - 100 µL of supernatant from each hybridoma to a separate well on a new 96-well plate and analyze using an appropriate assay to screen for antibodies against the antigen of interest (e.g. ELISA, flow cytometry, or Western blotting).
2. Expand the hybridomas that showed a positive response in the preliminary screening in step 1, as follows:
   i. Transfer 100 µL of cells to a 24-well plate containing 1 mL/well of Medium E or AOF Expansion & Cloning Medium.
   ii. Incubate plates at 37°C and 5% CO_2 for 3 - 4 days to expand preliminary positive hybridomas.
3. Perform confirmation testing of the preliminary positive hybridomas as described in step 1.
4. Expand the hybridomas that were confirmed to have a positive response in step 3, as follows:
   i. Transfer 500 - 600 µL of cells from the 24-well plate to a T-25 cm^2 flask containing 5 mL of Medium E or AOF Expansion & Cloning Medium (for cryopreservation)
   ii. Transfer 300 - 400 µL of cells from the 24-well plate to a 6-well plate containing 3 mL/well of Medium E or AOF Expansion & Cloning Medium (for further testing).
   iii. Incubate at 37°C and 5% CO_2 for 3 - 4 days to expand confirmed positive hybridomas.
5. When cells have grown to a suitable density (i.e. 0.5 - 1 x 10^6 cells/mL):
   i. T-25 cm^2 flask: Cryopreserve cells (see Appendix IV).
   ii. 6-well plate: Passage cells as described below:
      - Transfer 300 - 400 µL of cells from one well to one well of a new 6-well plate containing 3 mL of Medium E or AOF Expansion & Cloning Medium per well. Incubate at 37°C and 5% CO_2.
      - To transition cells from Medium E to AOF Expansion & Cloning Medium, no adaptation is required. Simply transfer 300 - 400 µL of cells into AOF Expansion & Cloning Medium.
      - To transition cells from Medium E to Medium A:
        - Transfer 300 - 400 µL of cells from one well to one well of a new 6-well plate containing 1.5 mL of Medium A and 1.5 mL of Medium E per well. Incubate at 37°C and 5% CO_2.
        - As a backup, continue culturing a sample of cells in Medium E until growth is confirmed in the cultures being adapted to Medium A.
        - To fully adapt the cells to Medium A, perform the next passage in 100% Medium A. Continue to maintain stable hybridoma cell lines in 100% Medium A, passaging every 3 - 4 days.

   Note: If subcloning is required, it is recommended to maintain culture in Medium E or AOF Expansion & Cloning Medium. Only transition to Medium A once monoclonal cultures have been established. For cells selected in semi-solid medium, monoclonality may be achieved as early as this stage but subcloning may be desired. For cells selected using liquid medium, subcloning is required (section 4.7).
6. Stable hybridoma cell lines can be expanded in T-25 cm^2 or T-75 cm^2 flasks for antibody production or for cryopreservation to secure the supply of the hybridoma.

4.7 Subcloning Hybridomas

For hybridomas generated using liquid culture medium, subcloning is required to achieve monoclonality. For hybridomas generated using semi-solid medium, subcloning may be necessary. Subcloning is recommended if a culture is suspected to be non-clonal (e.g. if the cell density in the plates was high and it is possible that cells of two or more colonies have been harvested).

Subcloning is also recommended for hybridomas that have been in continuous culture for an extended period of time, in particular if antibody production has declined and selection of high antibody-secreting subcultures is desired. Subclone hybridomas in semi-solid or liquid media as described below.

4.7.1 Subcloning Using Semi-Solid Medium

Recommended media for semi-solid subcloning include ClonaCell™-HY Medium D or ClonaCell™-HY Medium D without HAT.

1. Culture the hybridomas in 10 mL of ClonaCell™-HY Medium E until a maximum cell density of 2×10^5 cells/mL is reached.
2. Prepare a cell suspension at a density of 100 cells/mL in Medium E.
3. In a 15 mL conical tube, mix 9 mL of Medium D and 1 mL of hybridoma cell suspension prepared in step 2. Incubate at 37°C for 15 minutes to allow air bubbles to rise to the surface.
4. Plate the suspension in one Petri dish as indicated in section 4.4.1, step 5. Incubate at 37°C and 5% CO_2 for 10 - 14 days.
5. Examine the dish for the presence of colonies visible to the naked eye. Assuming a plating efficiency of 50 - 80%, there will be 50 - 80 colonies on the plate.
6. Remove 15 - 20 colonies from the dish using a pipettor set to 10 µL. Pipette each clone into an individual well of a 96-well tissue culture plate containing 200 µL/well of Medium E or AOF Expansion & Cloning Medium.
7. Incubate the plates at 37°C and 5% CO_2 for 3 - 4 days. Do not let cells overgrow.
8. Harvest subcloned colonies (section 4.5.1), then screen and expand colonies as described in section 4.6.

4.7.2 Subcloning Using Limiting Dilution Assay (LDA) in Liquid Medium

Recommended media for LDA include ClonaCell™-HY Medium E, ClonaCell™-HY AOF Expansion & Cloning Medium, or ClonaCell™-HY Liquid HAT Selection Medium.

Round-bottom 96-well plates are recommended in this protocol. Cells roll to the bottom of the well and can be directly identified by microscope to determine if there is more than one cell per well; this is not possible with a flat-bottom 96-well plate.

1. (Optional) The day before subcloning, perform a half-medium change (100 µL) with fresh medium.
2. Perform a viable cell count of hybridoma cells to be subcloned using Trypan Blue and a hemocytometer or an automated cell counter.
3. To plate at a limiting dilution cell density of 0.5 - 1 cell/well, dilute the hybridoma cells to 2.5 - 5 viable cells/mL in desired medium in a total volume of 20 mL per LDA plate. It is recommended to plate 2 - 3 LDA plates per subcloned hybridoma.

   For example, for 2 plates, prepare 40 mL of 2.5 - 5 cells/mL suspension in desired medium.
4. Mix by inverting the tube several times.

5. Transfer the diluted cells to a reservoir. Using a multi-channel pipettor, aseptically transfer 200 µL of the 2.5 - 5 cells/mL suspension to each well of a round-bottom 96-well plate.
6. Incubate plates in a humidified incubator at 37°C and 5% CO_2 for 7 - 14 days.

   (Optional) After 1 hour, remove the plates from the incubator and examine each well using an inverted microscope. At this stage, it is often possible to identify wells that contain more than one cell. These cells are not monoclonal; place an X on the cover of the dish over those wells so that they are excluded from subsequent screening and expansion.
7. Harvest subcloned colonies (section 4.5.2), then screen and expand colonies as described in section 4.6.

5.0 Troubleshooting

The following sections include some of the most common problems associated with the generation, selection, and cloning of hybridomas for the purpose of producing monoclonal antibodies, along with suggested causes.

5.1 Low Number of Hybridomas After Fusion and Selection

Typically a good fusion will yield over 1000 clones. A considerably lower number of hybridomas may be the result of the following:

- *Poor growth or low viability of myeloma cells prior to fusion*. Poor growth may occur if myeloma cells have not been sufficiently adapted to Medium A or are used too soon after initiating culture from cryopreserved cells. Low hybridoma viability may also occur if myeloma cell density is too high on the day of the fusion. See section 4.1 for further information.
- *Myeloma cells are contaminated with mycoplasma*. Mycoplasma consume thymidine, which can result in low numbers of recovered hybridomas during HAT selection. Test parental myeloma cells for mycoplasma prior to fusion. If myeloma cells are positive for the presence of mycoplasma, do not proceed with fusion; thaw a new vial or obtain new myeloma cells.
- *Poor viability of spleen cells prior to fusion*. There may be several causes of this problem, including the age and health status of the immunized mouse and the time period between spleen harvesting and fusion. It is recommended to work quickly and perform the fusion as soon as possible (preferably within 1 hour) after isolating the spleen cells. Myeloma cells should also be used as soon as possible after harvesting.
- *Poor viability of the fused cells*. Freshly fused cells are fragile and should be 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.

PEG-mediated fusion

- *Serum was not efficiently removed from the cells prior to the fusion* (see sections 4.1 and 4.2). 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 and prior to the addition of PEG (see section 4.3.2).
- 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.

5.2 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 is recommended for immunization.
- *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. See Appendix I for a suggested immunization schedule.
- *Poor sensitivity of the screening assay*. The screening assay should target antibodies that specifically bind the antigen of interest. Antigens used for screening may be degraded, not in a native conformation, or blocked, affecting the ability of the assay to detect antibodies that bind the target antigen.

5.3 Diffuse or Runny Colonies in Semi-Solid Medium

- *Disturbing the dishes before Day 10*. Methylcellulose is a viscous solution, and disturbing the dishes will break apart the small colonies forming and cause them to appear diffuse or runny. Do not touch dishes for the first 10 days.
- *Low viscosity of Medium D due to improper thawing* will result in diffuse or runny colonies. Medium D should not be thawed in a water bath, as the water is not of uniform temperature. Heating Medium D above 37°C can cause the methylcellulose to precipitate, lowering the viscosity of the medium.

5.4 Low Cloning Efficiency of Positive Hybridomas

- *Poor viability or low growth rate of the hybridoma and/or inherent genetic instability of the cells at the time of cloning* may result in very few growing or expressing hybridomas being obtained after cloning. If positive clones can still be identified, immediately subclone the hybridoma to preserve the cell line's stability and clonality. Cells should be in logarithmic growth phase and have > 90% viability prior to cloning for optimal cloning efficiency.

5.5 Difficulty Picking Individual Colonies

- If hybridoma colonies in Medium D (section 4.4.1) are not discrete or well distributed because the cell density is too high, or the colonies are diffuse or runny due to low viscosity of the medium, plucking individual colonies may be difficult. In this case, subclone positive hybridomas as soon as possible after the cells have been expanded in Medium E (section 4.6). Follow a protocol for subcloning in section 4.7.

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Appendix I: Mouse Immunization

Mice are typically immunized 6 - 10 weeks before performing fusions to enable a robust immune response to develop against the antigen of interest before proceeding to generate hybridomas. This section provides an example of a typical protocol for immunizing BALB/c or C57BL/6 mice prior to fusion; the actual timing may vary depending on the antigen used, immunization route, and other factors.[8] It is desirable to immunize mice with a pure antigen, as this simplifies the screening of hybridomas. However, complex antigenic mixtures may also be used.

1. Collect a sample of serum or plasma prior to immunization to use as a baseline control for antibody screening. Follow institutionally approved methods for collecting blood samples from mice. Store the serum or plasma with 0.1% sodium azide at -20°C.
2. Inject 2 - 4 adult mice with each antigen. Typically 20 - 100 µg of purified antigen or 100 - 200 µg of antigen mixture is injected intraperitoneally (IP) in a total volume of 200 µL with adjuvant.

   Note: Preparation of a stable emulsion is critical to generate a strong immune response.
3. Repeat the injection 14 - 30 days later.
4. Collect a test bleed from each mouse 10 - 14 days after immunization. Prepare serum from the blood sample and assess antibody titer by ELISA, immunofluorescence, flow cytometry, immunoblotting, etc.

   Note: Be sure to compare with the pre-immune serum from the same animal.
5. Select the mouse with the highest antibody titers for further boosting with antigen. Continue to give injections at 2-week intervals until a good titer of antibody is obtained.
6. Four days before the day of fusion, perform a final boost of the selected mouse intraperitoneally (IP) or intravenously (IV) with 10 - 50 µg of antigen in saline without adjuvant in a maximum volume of 200 µL. Prepare to fuse spleen cells 3 - 4 days later.

Appendix II: Preparation of the Splenocyte Suspension

1. Sacrifice an immunized mouse and wash the fur with 95% ethanol. Clip fur, cut skin, and pull back to expose chest.
2. Remove the spleen and place in a sterile Petri dish containing 5 mL of ClonaCell™-HY Medium B. Trim off any large pieces of fatty tissue.
3. Disaggregate the spleen into a single-cell suspension.

   Note: There are various protocols for doing this. One suggestion is to transfer the spleen to a fine mesh screen placed on top of a 50 mL conical tube, and use the plunger of a 3 mL syringe to grind the cells through the screen. Rinse the screen with Medium B. Only the spleen membrane should be left in the screen. Gently pipette the cells up and down to disrupt clumps. Try not to introduce foam.

Appendix III: Alternative 96-Well Plate Format for Selection and Cloning of Hybridomas in ClonaCell™-HY Medium D

This method is an alternative to the standard cloning and selection method described in section 4.4 and is meant to reduce the need to harvest and expand large numbers of hybridoma colonies before screening. The main difference between this method and the standard method is that the fusion products, suspended in semi-solid ClonaCell™-HY Medium D, are plated into individual wells of 96-well plates instead of 100 mm culture dishes. Hybridomas develop in the semi-solid layer as discrete colonies and secrete antibodies into the surrounding medium. Liquid medium (ClonaCell™-HY Medium E) is layered onto the semi-solid layer, and the secreted antibodies in the semi-solid layer diffuse into the liquid medium. The liquid medium can be harvested and tested for specific antibodies without disturbing the hybridoma colonies in the semi-solid medium underneath. The main advantage over the standard format is that individual colonies are tested for specific antibody production without the need to harvest and expand every colony first. Only positive colonies need to be plucked, resulting in considerable savings in time and labor. See the procedure diagram on page 22 for an overview.

Follow the protocols described in sections 4.1 - 4.2, then proceed as follows:

1. On the day of the fusion, place ClonaCell™-HY Medium D at 2 - 8°C to thaw overnight.
2. Perform a fusion of myeloma cells and splenocytes as described in section 4.3.1 (electrofusion) or section 4.3.2 (PEG-mediated fusion).
3. Incubate the fusion products at 37°C and 5% CO_2 in ClonaCell™-HY Medium C for 16 - 24 hours.
4. On the day after the fusion, vigorously shake the thawed Medium D to mix the contents of the bottle and warm to room temperature (15 - 25°C).
5. Determine the optimal number of cells to plate per well in order to obtain one colony per well. We recommend plating 10,000 - 80,000 cells/well.

   Note: If you already have experience with hybridoma selection in liquid HAT medium, plate the same number of cells per well in the semi-solid medium as you would in liquid medium. Resuspend the cell suspension in Medium C for a total volume of 10 mL. It is critical to not exceed the 10 mL final volume. If you wish to add additional cytokines or growth factors to Medium D, include this volume in the total 10 mL resuspension volume.
6. Combine 10 mL of fused cell suspension with 90 mL of Medium D. Gently invert the bottle 6 times to mix thoroughly and let sit for 15 minutes to allow bubbles to rise to the surface.
7. Using either a multi-channel pipettor and sterile wide-bore pipette tips or a repeater pipette with appropriate tips (e.g. Gilson Catalog #F164001 and F164140, respectively), dispense 60 - 80 µL of Medium D into each well of 96-well plates. This will yield between 12 - 16 plates depending on the volume plated.

   Note: Medium D is a viscous solution and is therefore difficult to pipette accurately. However, it is not critical to dispense exactly the same volume into each well.
8. Incubate the plates at 37°C and 5% CO_2. The incubator should be well humidified to prevent excessive evaporation.

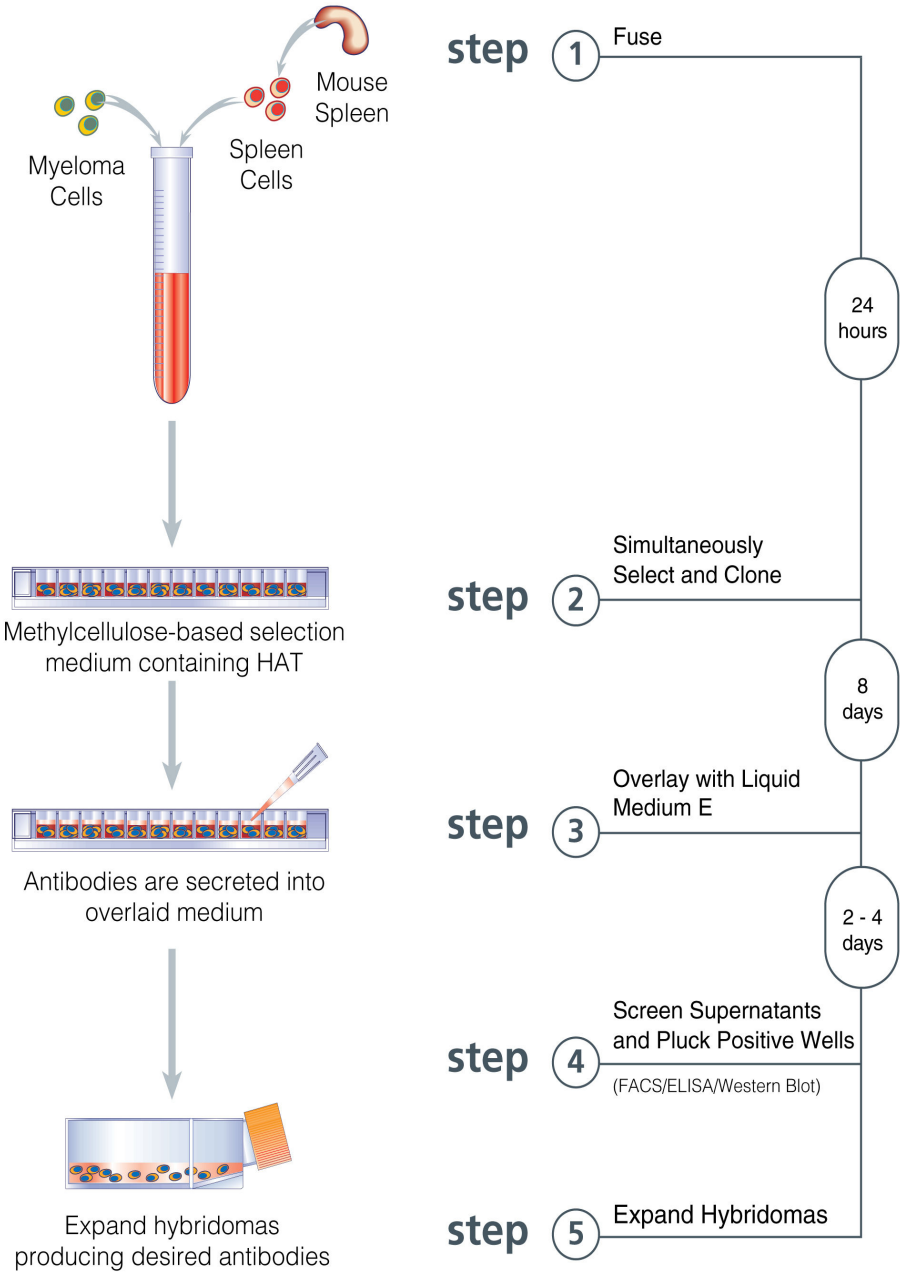
   Note: If desired, the plates may be placed inside a plastic container (e.g. Corning® 245 mm Square Dish, Non-Treated [Catalog #38020]) along with an open Petri dish containing distilled water. If using a regular storage container, ensure that the lid is loose to facilitate gas exchange.

9. Following 8 days of undisturbed incubation, examine wells for the presence of colonies and gently overlay 150 µL of warm (37°C) ClonaCell™-HY Medium E onto the semi-solid medium of each well containing colonies. Alternatively, all wells may be overlaid with 150 µL of warm (37°C) Medium E regardless of the presence of colonies, and analysis performed on all wells.
10. Incubate the plates for an additional 2 - 4 days at 37°C and 5% CO_2.

    Note: The overlay incubation time may be increased further to ensure the detection of low-expressing hybridomas.
11. Carefully remove 50 - 100 µL of the overlaid Medium E without disturbing the colonies in the semi-solid medium. Test the supernatants for specific antibodies using an assay appropriate for the antigen of interest (e.g. ELISA, flow cytometry, Western blotting, etc.).
12. The contents of wells that tested positive for antibodies against the antigen of interest should be gently resuspended in the medium in the well. To expand the hybridomas, transfer to wells of a 24-well plate containing 1 mL of Medium E.

    Note: If a well contains more than a single colony, it may be possible to harvest these clones separately and transfer them to individual wells for expansion and retesting to determine which clone produces the antibody of interest. If wells contain more than one colony and harvesting of individual colonies is not possible, the hybridomas need to be subcloned either immediately after harvesting or after a brief 1- to 2-day recovery and expansion period in Medium E. Subcloning is not necessary for positive clones harvested from wells containing only a single hybridoma or for individual hybridomas harvested from wells containing more than one colony, as these hybridomas should already be monoclonal. However, it may still be useful to subclone these hybridomas to select for stable high-producing subclones.

ClonaCell™-HY 96-Well Procedure Diagram



Appendix IV: Cryopreserving and Thawing Cells

Cryopreserving Hybridomas

Cryopreserve cells at a concentration of 2 - 5 x 10^6 cells per cryovial. Use the cryopreservation medium CryoStor® CS10 (Catalog #07930) as described in the Product Information Sheet (Document #29941), available at www.stemcell.com or contact us to request a copy. Alternatively, follow the procedure below.

1. Label the required number of sterile 2 mL cryovials (1.8 mL capacity).
2. Prepare a 20% DMSO solution in fetal bovine serum (FBS) as follows:
   i. Place FBS in a 15 mL conical tube and cool on ice. Slowly add appropriate volume of DMSO and mix well.
   ii. Filter-sterilize solution using a 0.2 µm filter and keep on ice.
3. Harvest cells and resuspend in cold (2 - 8°C) FBS at twice the desired final cell concentration.

   For example, resuspend to 4 x 10^6 cells/mL for cells cryopreserved at 2 x 10^6 cells/cryovial.
4. Slowly add the 20% DMSO in FBS solution (prepared in step 2) at a ratio of 1:1 to the tube containing the cells in FBS. Continue to mix during the addition. Transfer 1 mL of cells in freezing medium to each cryovial.

   Note: The final cell suspension will be in 90% FBS:10% DMSO.
5. Immediately place cryovials into freezing containers.

   Note: To ensure good viability and cell recovery, do not let cells sit in freezing medium at room temperature. Keep on ice and transfer within 15 minutes to the freezing container. Handle freezing container according to manufacturer's instructions.
6. Place freezing container containing cryovials in -70°C or -135°C freezer overnight.
7. Remove frozen cryovials from the freezing container and store at -70°C or -135°C for short-term storage or liquid nitrogen for long-term storage.

Thawing and Culturing Cells (Parental Myeloma Cells, Hybridomas)

1. Warm ClonaCell™-HY Medium E or ClonaCell™-HY AOF Expansion & Cloning Medium in a 37°C water bath.
2. Remove cryovial from storage and thaw cells quickly by agitating the cryovial in a 37°C water bath.
3. Draw up the cell suspension in a 2 mL pipette and place in a 15 mL conical tube. Slowly add 10 mL of Medium E or AOF Expansion & Cloning Medium dropwise to the cells. Centrifuge at 400 x *g* for 10 minutes at room temperature (15 - 25°C) or 37°C.

   Note: This wash step is required to remove DMSO.
4. Discard the supernatant and resuspend the cells in 1 - 2 mL of Medium E or AOF Expansion & Cloning Medium.
5. Determine the viable cell concentration using a hemocytometer or an automated cell counter.

6. Seed culture flask at a cell density of ~5 x 10^4 viable cells/mL. Add cells and Medium E or AOF Expansion & Cloning Medium to the desired culture flask as follows:
   - T-25 cm^2 flask: 5 mL final volume
   - T-75 cm^2 flask: 25 mL final volume
7. Incubate flasks at 37°C and 5% CO_2.
8. Grow cells to ~70 - 80% confluence (or to the cell density suggested by the protocol) and passage cells every 2 - 4 days. Once cells are growing well (viability is > 90% for 1 passage), cells may be cultured as follows:
   - For cells in Medium E: If desired, cells can be transitioned to Medium A or AOF Expansion & Cloning Medium.
   - For cells in AOF Expansion & Cloning Medium: Continue to culture in AOF Expansion & Cloning Medium.

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