

TECHNICAL NOTE

CLONACELL™-HY 96-WELL PLATE SELECTION AND CLONING

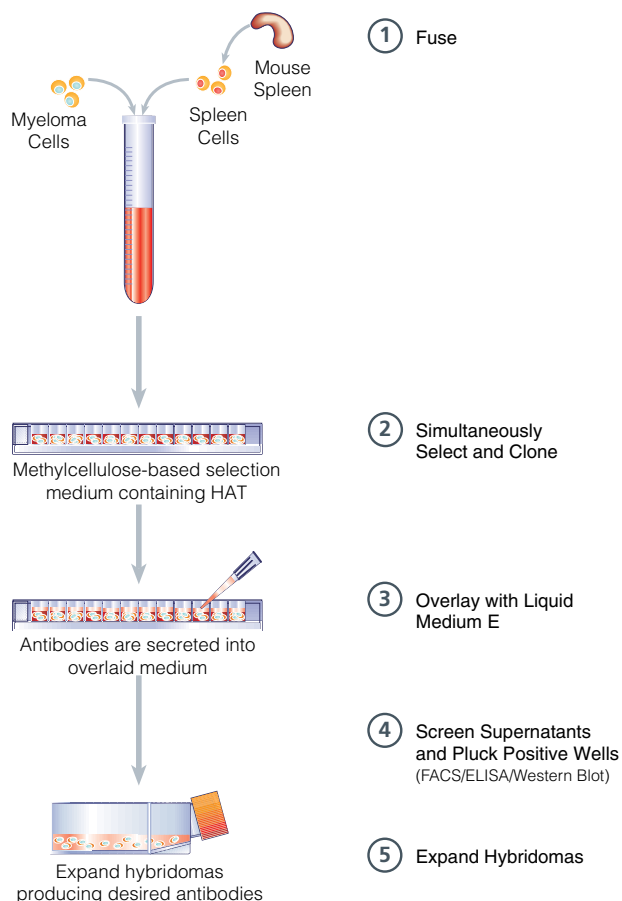
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Background

Conventional methods to select and clone monoclonal antibody-producing hybridomas involve multiple dilution steps in liquid medium. STEMCELL Technologies provides a methylcellulose-based semi-solid medium, ClonaCell™-HY Medium D, which combines the hybridoma selection and cloning steps, reducing the overall time necessary to produce monoclonal antibodies by up to 26 days. The standard protocol for selection and cloning of hybridomas in ClonaCell™-HY media involves plating the fused cell suspension (i.e. fused myeloma and splenocytes) into the selective semi-solid medium in 10 cm plates. Hybridomas grow to form colonies which are picked from the semi-solid medium after 10 - 14 days of incubation. Colonies are transferred to wells of a 96-well plate and cultured in liquid medium prior to screening supernatants for positive clones (refer to Figure 1 on page 312).

The following technical note describes the use of semi-solid methylcellulose-based medium (ClonaCell™-HY Medium D) and 96-well plates to reduce the need to harvest and expand large numbers of hybridoma colonies before screening (Figure 1 on this page). A fused cell suspension mixed with ClonaCell™-HY Medium D is plated directly into individual wells of a 96-well plate. Hybridomas develop in the semi-solid medium as discrete colonies and secrete antibodies into the surrounding medium. Liquid medium is layered over the semi-solid medium and the secreted antibodies diffuse into the liquid medium. The liquid medium is then harvested and screened for specific antibodies. In this method, colonies can be tested for secretion of specific antibodies without the need to harvest and expand every colony first. Therefore, only positive colonies need to be plucked, resulting in considerable time and labor savings.

FIGURE 1. ClonaCell™-HY 96-Well Procedure



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Protocol

It is recommended that a fresh vial of myeloma cells be thawed at least one week prior to the expected fusion date (Note: ensure myeloma cells are mycoplasma-free). Ideally, the cells used for fusion should be in their logarithmic growth phase.

1. On the day of fusion, place ClonaCell™-HY Hybridoma Selection Medium, Medium D (Catalog #03804), at 2 - 8°C to thaw overnight. Do not place medium in a water bath to thaw.
2. Perform fusion of myeloma cells and splenocytes to yield between 10 - 80 million fused hybridoma cells. For a more detailed fusion protocol, please refer to the ClonaCell™-HY Technical Manual (available at www.stemcell.com).
3. Incubate the fused cells for 16 - 24 hours in ClonaCell™-HY Recovery Medium C (Catalog #03803), at 37°C in a humidified, 5% CO₂ incubator.
4. On the day after 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 to arrive at 1 colony per well. We recommend a range of 10,000 - 80,000 cells/well. If you already have experience with hybridoma selection in liquid HAT medium, plate the same number of cells/well in the semi-solid medium as you would in liquid medium.
6. 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 volume the cells are being resuspended in.
7. Combine 10 mL of fused cell suspension with 90 mL Medium D. Gently invert the bottle 6 times to mix thoroughly and let sit for 15 minutes to allow the bubbles to surface.
8. Using either a multi-channel pipettor and sterile pipette tips (the use of wide-bore pipette tips is recommended), or a repeat pipettor and sterile syringe, dispense 60 - 80 µL of Medium D into each well of a 96-well plate. This will yield between 12 - 16 plates depending on the volume plated. Medium D is a viscous solution and therefore difficult to pipette accurately; however, it is not critical to dispense exactly the same volume into each well.
9. Incubate the plates at 37°C in a humidified, 5% CO₂ incubator. The incubator should be well humidified to prevent excessive evaporation. If desired, the plates may be placed inside a plastic container that allows proper gas exchange (e.g. 245 mm x 245 mm; Catalog #27140/27141) along with an open Petri dish containing sterile water.
10. Following 8 days of undisturbed incubation, examine wells for the presence of colonies by eye or microscope and gently overlay 150 µL of prewarmed (to 37°C) ClonaCell™-HY Hybridoma Growth Medium, Medium E (Catalog #03805), onto the semi-

solid medium of each well containing colonies. Alternatively, all wells may be overlaid with 150 µL of prewarmed Medium E regardless of the presence of colonies and analysis performed on all wells.

11. Incubate plates for an additional 2 - 4 days at 37°C in a humidified, 5% CO₂ incubator. The overlay incubation time may be increased further to ensure the detection of low-expressing hybridomas.
12. Carefully remove 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 system appropriate for the antigen involved (e.g. ELISA, flow cytometry, Western Blotting).
13. The contents of wells that tested positive for antibodies against the antigen of interest should be gently resuspended and transferred to a single well of a 24-well plate containing 1 mL of Medium E, to expand the hybridomas. 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. If wells contain more than one colony, and harvesting of individual colonies is not possible, the hybridomas need to be recloned either immediately after harvesting or after a brief 1 - 2 days recovery and expansion period in Medium E. Recloning is not necessary for positive clones which can be harvested independently as these hybridomas should already be monoclonal. However, it is useful to reclone these hybridomas when selecting for stable high producing subclones.

For additional details on hybridoma generation please refer to the Technical Manual for ClonaCell™-HY Hybridoma Cloning Kit (Manual Catalog #28411), available at www.stemcell.com.

Time Comparison of Traditional, ClonaCell™-HY, and ClonaCell™-HY 96-well Protocols for Hybridoma Production

LIQUID MEDIA	TIME (DAYS)*	CLONACELL™-HY	TIME (DAYS)*	CLONACELL™-HY 96-WELL PROTOCOL	TIME (DAYS)*
Fuse	1	Fuse	1	Fuse	1
Selection and Cloning by Limiting Dilution	14	Selection and Cloning in 10 cm Dish	10-14	Selection and Cloning in 96 Well Plates	8
Screening	1	Colony Isolation	1	Overlay with Medium E	2-4
Subcloning by Limiting Dilution	14	Expansion	2-4	Screening and Isolating Colonies (Monoclonal Hybridomas)	1
Screening	1	Screening	1		
Total Days	31	Total Days	15-21	Total Days	12-14

*Estimated times will vary depending on volume of work.