

ClonaCell®-HY  
Hybridoma Cloning Kit

For Research Use Only. Not For Therapeutic or Diagnostic Use.

---

**In North America**

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. [info@stemcell.com](mailto:info@stemcell.com)  
E. [orders@stemcell.com](mailto:orders@stemcell.com)

**In Europe**

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. [info.eu@stemcell.com](mailto:info.eu@stemcell.com)

**In Australia**

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. [info.aus@stemcell.com](mailto:info.aus@stemcell.com)

**In Singapore**

T. 65.972.66660  
E. [info.sg@stemcell.com](mailto:info.sg@stemcell.com)

**STEMCELL Technologies**

Version 2.2.0  
June 2009  
Catalog #28411

## Table of Contents

|            |   |           |
|------------|---|-----------|
| <b>1.0</b> | <b>Introduction</b> .....   | <b>1</b>  |
| 1.1        | General Background.....   | 1         |
| 1.2        | Description.....  | 2         |
| 1.3        | ClonaCell®-HY Products.....   | 4         |
| 1.4        | Additional Equipment, Reagents, and Supplies Required.....  | 4         |
| <b>2.0</b> | <b>Methods</b> .....  | <b>6</b>  |
| 2.1        | Mouse Immunization.....   | 6         |
| 2.2        | Myeloma Cells.....  | 6         |
| 2.3        | Splenocytes.....  | 7         |
| 2.4        | Fusion.....   | 7         |
| 2.5        | Selection and Cloning.....  | 8         |
| 2.6        | Harvest.....  | 9         |
| <b>3.0</b> | <b>Troubleshooting</b> .....  | <b>10</b> |
| 3.1        | Low Number of Hybridomas After Fusion and Selection.....  | 10        |
| 3.2        | No or Too Few Positive Hybridomas.....  | 10        |
| 3.3        | Hazy or Runny Colonies.....   | 11        |
| 3.4        | Other.....  | 11        |
| <b>4.0</b> | <b>Appendix</b> .....   | <b>12</b> |
| 4.1        | Appendix I: Immunization of BALB/c Mice.....  | 12        |
| 4.2        | Appendix II: Preparation of the Splenocyte Suspension.....  | 13        |
| 4.3        | Appendix III: Alternative 96-well Plate Format for the Selection and Cloning of Hybridomas in ClonaCell®-HY Medium D..... | 13        |
| 4.4        | Appendix IV: Recloning in ClonaCell®-HY.....  | 16        |
| 4.5        | Appendix V: Freezing and Thawing Cells.....   | 16        |
| 4.5.1      | Freezing Hybridomas.....  | 16        |
| 4.5.2      | Thawing and Culturing of Cells (Parental Myeloma Cells, Hybridomas).....  | 17        |
| <b>5.0</b> | <b>References</b> .....   | <b>18</b> |

For Research Use Only. Not For Therapeutic or Diagnostic Use.

### In North America

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. info@stemcell.com  
E. orders@stemcell.com

### In Europe

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. info.eu@stemcell.com

### In Australia

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. info.aus@stemcell.com

### In Singapore

T. 65.972.66660  
E. info.sg@stemcell.com

### STEMCELL Technologies

Version 2.2.0  
June 2009  
Catalog #28411



## 1.0 Introduction

### 1.1 General Background

George Köhler and César Milstein described the first derivation of monoclonal antibodies (mAbs) of defined specificity in 1975<sup>1</sup> and, for their work, were awarded the Nobel Prize in Physiology and Medicine in 1984. A variety of methods have been used to fuse, grow, select, and clone hybridomas since the original publication.<sup>2-7</sup> However, many of the approaches that are used have limitations; they are time consuming and require many manipulations. A problem often encountered is that some hybridomas overgrow others prior to cloning. Often, these faster growing cells do not synthesize antibodies<sup>8</sup>, resulting in a failure to obtain the desired hybridomas. A solution to this problem is to clone the hybridomas, as soon as possible, after fusion using agar-based<sup>5</sup>, agarose-based<sup>6</sup>, or methylcellulose-based<sup>4</sup> semi-solid media.

ClonaCell<sup>®</sup>-HY is a methylcellulose-based formulation containing growth factors and medium supplements optimized to support the selection and growth of hybridoma clones. The ClonaCell<sup>®</sup>-HY method is designed to select and clone hybridoma cells soon after fusion. This eliminates the possibility of overgrowth of potentially valuable slow-growing clones by fast-growing clones. In addition, the number of clones to be screened for the secretion of a specific antibody is minimized since identical daughter cells are not produced prior to the direct cloning. 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.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

#### In North America

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. [info@stemcell.com](mailto:info@stemcell.com)  
E. [orders@stemcell.com](mailto:orders@stemcell.com)

#### In Europe

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. [info.eu@stemcell.com](mailto:info.eu@stemcell.com)

#### In Australia

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. [info.aus@stemcell.com](mailto:info.aus@stemcell.com)

#### In Singapore

T. 65.972.66660  
E. [info.sg@stemcell.com](mailto:info.sg@stemcell.com)

#### STEMCELL Technologies

Version 2.2.0  
June 2009  
Catalog #28411

## 1.2 Description

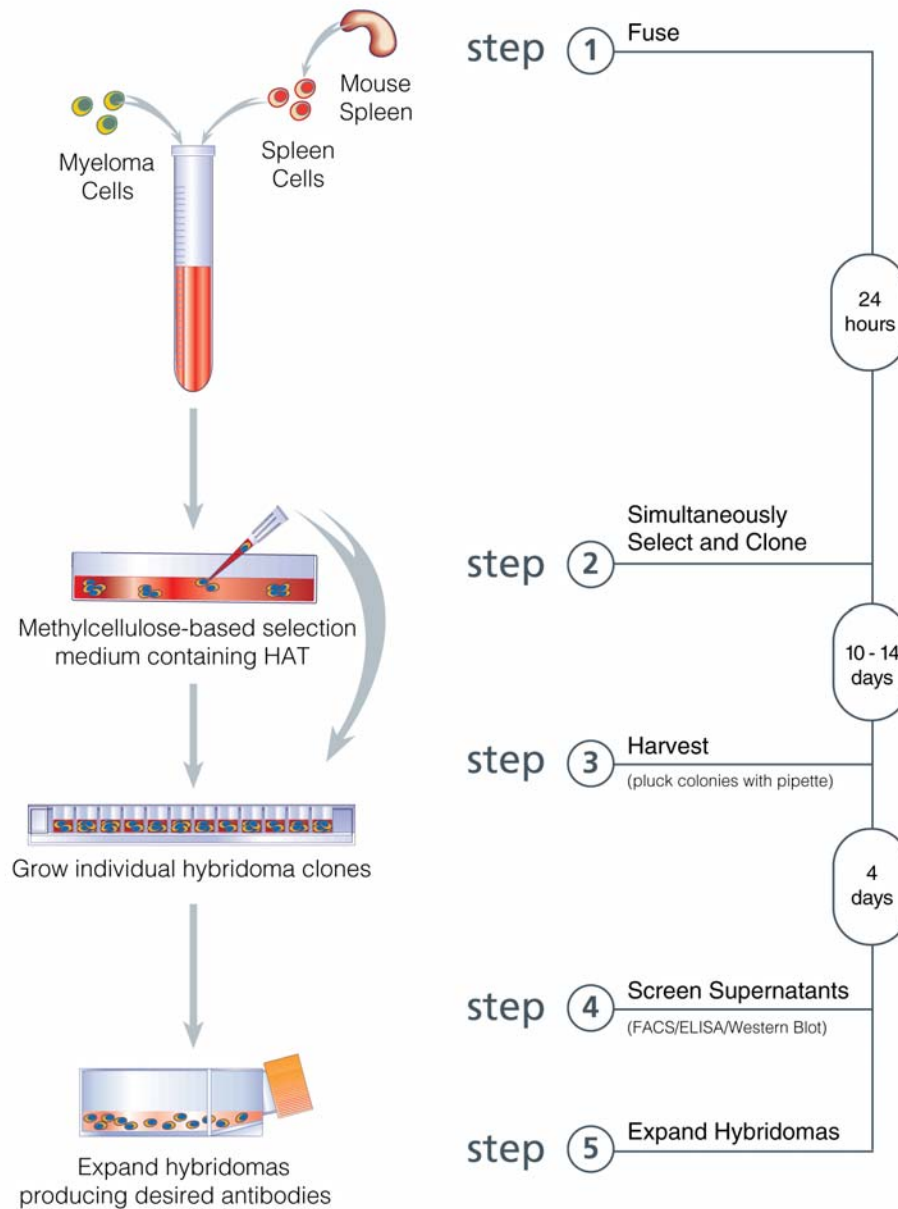
ClonaCell<sup>®</sup>-HY is a special formulation of methylcellulose-based medium containing growth factors, B-cell stimulators and medium supplements optimized for the growth of single cells. Advantages of ClonaCell<sup>®</sup>-HY over standard hybridoma selection and cloning methods:

- HAT selection and cloning of hybridomas are performed in one step, minimizing both the time and the materials required.
- Fewer manipulations are required; therefore the possibility of culture contamination is greatly reduced.
- Large numbers of hybridomas can be selected and tested. More than a thousand clones can be grown in ten 100 mm petri dishes in a single step.
- Growth conditions have been optimized to give high plating efficiency ensuring maximum hybridoma yield.
- Direct cloning prevents the overgrowth of potentially valuable slow-growing clones.
- Hybridoma colonies are monoclonal from the start, so recloning is not necessary.
- Plating of the initial fusion mixture and simultaneous cloning are performed in minutes vs. hours when using methods involving suspension cultures and limiting dilutions.
- ClonaCell<sup>®</sup>-HY minimizes the number of clones to be screened for antibody secretion because all daughter cells are found in the same colony.
- Estimated savings of 11 - 26 days and 30 - 50% of technician's work hours.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

| In North America   | In Europe   | In Australia   | In Singapore                               | STEMCELL Technologies                        |
|--|---|--|--|--|
| Toll-Free T. 1.800.667.0322<br>Toll-Free F. 1.800.567.2899<br>T. 1.604.877.0713<br>F. 1.604.877.0704<br>E. info@stemcell.com<br>E. orders@stemcell.com | Toll-Free T. 00.800.7836.2355<br>Toll-Free F. 00.800.7836.2300<br>T. +33 (0)4.76.04.75.30<br>F. +33 (0)4.76.18.99.63<br>E. info.eu@stemcell.com | Toll-Free T. 1.800.060.350<br>F. +61 (03)9338.4320<br>E. info.aus@stemcell.com | T. 65.972.66660<br>E. info.sg@stemcell.com | Version 2.2.0<br>June 2009<br>Catalog #28411 |

**Figure 1. ClonaCell-HY Procedure Overview**



For Research Use Only. Not For Therapeutic or Diagnostic Use.

**In North America**

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. [info@stemcell.com](mailto:info@stemcell.com)  
E. [orders@stemcell.com](mailto:orders@stemcell.com)

**In Europe**

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. [info.eu@stemcell.com](mailto:info.eu@stemcell.com)

**In Australia**

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. [info.aus@stemcell.com](mailto:info.aus@stemcell.com)

**In Singapore**

T. 65.972.66660  
E. [info.sg@stemcell.com](mailto:info.sg@stemcell.com)

**STEMCELL Technologies**

Version 2.2.0  
June 2009  
Catalog #28411

### 1.3 ClonaCell®-HY Products

| Product                               | Volume | Description   | Contains  | Catalog # |
|---------------------------------------|--------|---|---|-----------|
| <b>Medium A</b> (Pre-fusion)          | 500 mL | Myeloma growth medium and Hybridoma expansion medium.                     | Dulbecco's Modified Eagle's Medium (DMEM), pre-selected serum, gentamycin, and supplements. | 03801     |
| <b>Medium B</b> (Fusion)              | 500 mL | Medium used to wash cells prior to cell fusion and for use during fusion. | DMEM and gentamycin.  | 03802     |
| <b>Medium C</b> (Recovery)            | 100 mL | Fusion recovery medium to promote hybridoma viability.                    | DMEM, pre-selected serum, gentamycin, and supplements.                                      | 03803     |
| <b>Medium D</b> (Selection & Cloning) | 90 mL  | Semi-solid HAT hybridoma selection medium.                                | DMEM, methylcellulose, pre-selected serum, HAT, gentamycin, and supplements.                | 03804     |
| <b>Medium E</b> (Growth)              | 500 mL | Hybridoma growth medium.  | DMEM, pre-selected serum, HT, gentamycin, and supplements.                                  | 03805     |
| <b>Polyethylene Glycol (PEG)</b>      | 1.5 mL | Pre-tested solution for cell fusion.                                      | 50% solution of PEG in DMEM.  | 03806     |

### 1.4 Additional Equipment, Reagents, and Supplies Required

#### *Equipment*

- Biohazard safety cabinet certified for level II handling of biological materials
- Low speed bench centrifuge
- Microfuge
- 37°C incubator with humidity and gas control to maintain >95% humidity and an atmosphere of 5% CO<sub>2</sub> in air
- Pipette-aid
- Hemacytometer
- Routine light microscope
- Inverted microscope
- 37°C water bath
- Liquid nitrogen tank and freezing head
- Freezing container (i.e. "Mr. Frosty" Nalgene Catalog #5100); optional

For Research Use Only. Not For Therapeutic or Diagnostic Use.

#### **In North America**

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. info@stemcell.com  
E. orders@stemcell.com

#### **In Europe**

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. info.eu@stemcell.com

#### **In Australia**

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. info.aus@stemcell.com

#### **In Singapore**

T. 65.972.66660  
E. info.sg@stemcell.com

#### **STEMCELL Technologies**

Version 2.2.0  
June 2009  
Catalog #28411

*Reagents*

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

*Supplies*

- 1.5 mL sterile microcentrifuge tubes
- 50 mL sterile conical tubes
- 15 mL sterile conical tubes
- 25 mL sterile serological pipettes
- 10 mL sterile serological pipettes
- 5 mL sterile serological pipettes
- 2 mL sterile serological pipettes
- 1 mL sterile serological pipettes
- Pasteur pipettes - sterile
- T-25 cm<sup>2</sup> sterile tissue culture flask (Corning Catalog #430639, or equivalent)
- T-75 cm<sup>2</sup> sterile tissue culture flask (BD Catalog #353136, or equivalent)
- 100 mm sterile plastic disposable petri dishes
- 96-well sterile tissue culture plates (Catalog #27136)
- 24-well sterile tissue culture-treated plates (Corning Catalog #003526, or equivalent)
- 96-well ELISA plates (Nunc Catalog #537336, or equivalent)
- 12 mL syringe
- Blunt-end 16 gauge needle (Catalog #28110)
- Forceps (2)
- Fine scissors
- Fine-mesh metal screen or disposable cell strainer (Catalog #27305)
- Multi-channel pipettor, 8-channel or 12-channel, 20 - 200 µL
- Plastic container with a lid, large enough to hold ten 100 mm petri dishes

*Biologicals*

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

For Research Use Only. Not For Therapeutic or Diagnostic Use.

**In North America**

Toll-Free T. 1.800.667.0322  
 Toll-Free F. 1.800.567.2899  
 T. 1.604.877.0713  
 F. 1.604.877.0704  
 E. info@stemcell.com  
 E. orders@stemcell.com

**In Europe**

Toll-Free T. 00.800.7836.2355  
 Toll-Free F. 00.800.7836.2300  
 T. +33 (0)4.76.04.75.30  
 F. +33 (0)4.76.18.99.63  
 E. info.eu@stemcell.com

**In Australia**

Toll-Free T. 1.800.060.350  
 F. +61 (03)9338.4320  
 E. info.aus@stemcell.com

**In Singapore**

T. 65.972.66660  
 E. info.sg@stemcell.com

**STEMCELL Technologies**

Version 2.2.0  
 June 2009  
 Catalog #28411

## 2.0 Methods

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

### 2.1 Mouse Immunization

For immunization protocol, please refer to Appendix I, Section 4.1.

### 2.2 Myeloma Cells

The parental myeloma cells used to make the hybridoma 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. The parental myeloma cells should be mycoplasma free, fuse well and allow the formation of stable hybridomas that continually secrete specific monoclonal antibodies. Parental myeloma cells that meet these criteria (such as SP2/0 and X63Ag8.653) are widely available. Whenever possible, obtain a parental myeloma cell that has been proven to yield good stable hybridomas.

1. Thaw the parental myeloma cells (refer to Appendix V in Section 4.5.2) and culture in ClonaCell®-HY Pre-Fusion Medium (Medium A) for at least one week prior to fusion to ensure that the cells are well adapted to ClonaCell®-HY medium. Seed cells at a density of approximately  $5 \times 10^4$  cells/mL and passage every 2 days. The suggested maximum cell density is approximately  $4 \times 10^5$  cells/mL, although a cell density of up to  $8 \times 10^5$  cells/mL is acceptable.
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. If cells grow beyond  $8 \times 10^5$  cells/mL, passage them at least twice to return them to early-mid log phase growth prior to fusion.
4. Calculate the cell growth rate at every passage. The day before the fusion, count the viable cells and split so that at least  $2 \times 10^7$  parental myeloma cells will be available for fusion.
5. The recommended cell density for fusion is  $2 \times 10^5$  cells/mL. Only 100 mL of these cells is needed, but 200 mL should be cultured to ensure sufficient cell numbers for fusion.
6. Harvest the parental myeloma cells by centrifuging in a 50 mL conical centrifuge tube at room temperature (RT) or 37°C, at  $300 \times g$  (~1100 rpm) for 10 minutes. Wash 3 times by adding 30 mL of ClonaCell®-HY Fusion Medium (Medium B), and repeating the centrifugation. Remove the supernatant by pipette and resuspend the cell pellet in 25 mL of Medium B.
7. This step may be performed simultaneously with, or after, the spleen cell preparation 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.
8. Count live cells using viability stain (e.g. Trypan Blue, Catalog #07050). The viability of parental myeloma cells should be >95%.
9. Calculate the volume of cell suspension that contains  $2 \times 10^7$  viable cells. Keep cells at RT or 37°C until fusion (Section 2.4).

For Research Use Only. Not For Therapeutic or Diagnostic Use.

| In North America            | In Europe                     | In Australia               | In Singapore            | STEMCELL Technologies |
|-----------------------------|-------------------------------|----------------------------|-------------------------|-----------------------|
| Toll-Free T. 1.800.667.0322 | Toll-Free T. 00.800.7836.2355 | Toll-Free T. 1.800.060.350 | T. 65.972.66660         | Version 2.2.0         |
| Toll-Free F. 1.800.567.2899 | Toll-Free F. 00.800.7836.2300 | F. +61 (03)9338.4320       | E. info.sg@stemcell.com | June 2009             |
| T. 1.604.877.0713           | T. +33 (0)4.76.04.75.30       | E. info.aus@stemcell.com   |                         | Catalog #28411        |
| F. 1.604.877.0704           | F. +33 (0)4.76.18.99.63       |                            |                         |                       |
| E. info@stemcell.com        | E. info.eu@stemcell.com       |                            |                         |                       |
| E. orders@stemcell.com      |                               |                            |                         |                       |

## 2.3 Splenocytes

1. Disaggregate the spleen into a single cell suspension as described in Appendix II, Section 4.2. Wash splenocytes 3 times in 30 mL of Medium B, centrifuging at 400 x g (~1350 rpm) at RT or 37°C for 10 minutes each time and removing the supernatant by pipette. After the final wash resuspend the cells in 25 mL Medium B.
2. 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 1/10 dilution of cells in 3% Acetic Acid with Methylene Blue (Catalog #07060) (e.g. dilute 10 µL of the cell suspension with 90 µL of acetic acid).
4. Count cells in this diluted sample using a hemacytometer. Calculate the volume of cell suspension that contains  $1 \times 10^8$  cells. Place cells at room temperature or 37°C until fusion.

## 2.4 Fusion

Prepare PEG and media (Medium A, B, C) for fusion by prewarming to 37°C. If using fusion Method A (below), prepare a 37°C water bath.

1. Add  $2 \times 10^7$  parental myeloma cells and  $1 \times 10^8$  viable splenocytes (as calculated in Sections 2.2 and 2.3, respectively) to a 50 mL conical centrifuge tube and centrifuge for 10 minutes at 400 x g (~1350 rpm). Aspirate off supernatant.
2. Complete removal of the supernatant is essential to avoid dilution of PEG in the next step.
3. Fuse cells using one of the two methods outlined below:

### Method A

- a) Disrupt the cell pellet obtained in Section 2.4, Step 1 by gently tapping the bottom of the tube. The pellet must be disrupted for optimal fusion. Slowly add 1 mL of ClonaCell®-HY PEG Solution (PEG) to the pellet obtained in Section 2.4, Step 1 dropwise using a 1 mL pipette, over a period of 1 minute without stirring. Continually stir the cells gently, with the pipette tip, over the next minute.
- b) Add 4 mL Medium B to the fusion mixture, continuously stirring as before, over a period of 4 minutes.
- c) Slowly add 10 mL Medium B to the cells. Incubate for 15 minutes in water bath at 37°C.
- d) Slowly add 30 mL of Medium A and centrifuge the cells at 400 x g (~1350 rpm) for 7 minutes. Discard the supernatant and wash cells with 40 mL of Medium A to ensure that all PEG is removed.
- e) Slowly resuspend the cell pellet in 10 mL of ClonaCell®-HY Hybridoma Recovery Medium (Medium C). Transfer the cell suspension to a T-75 cm<sup>2</sup> tissue culture flask containing 20 mL of Medium C (total culture volume = 30 mL). Incubate for 16 - 24 hours at 37°C in 5% CO<sub>2</sub> atmosphere.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

#### In North America

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. info@stemcell.com  
E. orders@stemcell.com

#### In Europe

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. info.eu@stemcell.com

#### In Australia

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. info.aus@stemcell.com

#### In Singapore

T. 65.972.66660  
E. info.sg@stemcell.com

#### STEMCELL Technologies

Version 2.2.0  
June 2009  
Catalog #28411

## Method B

- a) Disrupt the cell pellet obtained in Section 2.4, Step 1 by gently tapping the bottom of the tube. Add 0.5 mL of ClonaCell<sup>®</sup>-HY PEG Solution (PEG) dropwise to the pellet using a 1 mL pipette. Centrifuge the mixture at 133 x g (~800 rpm) at RT or 37°C for 3 minutes. Aspirate off all PEG.

*It is important to completely break up the cell pellet prior to adding PEG in order to ensure efficient fusion of the cells. During this procedure, not all cells will form a pellet, as some will clump in the PEG. Do not aspirate the clumped cells. Work quickly since cells must not be exposed to PEG for too long or cell viability will drop.*

- b) Carefully add 5 mL of Medium B dropwise to the pellet while gently swirling the tube to resuspend the cells.
- c) Slowly add 5 mL of ClonaCell<sup>®</sup>-HY Hybridoma Recovery Medium (Medium C) to the solution. Continue to swirl the tube.
- d) Transfer the cell suspension to a T-75 cm<sup>2</sup> tissue culture flask containing 20 mL of Medium C (total culture volume = 30 mL). Incubate for 16 - 24 hours at 37°C in 5% CO<sub>2</sub> atmosphere.
- e) There will still be clumps of cells at this point which will dissolve overnight. Be gentle with these cells.

## 2.5 Selection and Cloning

1. On the day of the fusion, place ClonaCell<sup>®</sup>-HY Hybridoma Selection Medium (Medium D) at 2 - 8°C and thaw overnight. On the day after the fusion, shake vigorously to mix contents well and let warm to room temperature.

*It is not recommended to thaw Medium D in a water bath. Water is often not of uniform temperature and heating Medium D above 37°C can cause the methylcellulose to precipitate.*

2. Transfer fused cell suspension into a 50 mL conical tube and centrifuge for 10 minutes at 400 x g (~1350 rpm) at RT or 37°C. Remove the supernatant. Resuspend the cells in Medium C to a total volume of 10 mL.

*It is critical not to 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 volume that the cells are being resuspended in.*

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

3. Transfer the 10 mL cell suspension into the 90 mL of Medium D. Mix thoroughly by gently inverting the bottle several times. Let sit for 15 minutes at RT or 37°C to allow the bubbles to rise to the top.
4. Using a 12 mL syringe and 16 gauge blunt-end needle, aseptically plate out 9.5 mL of cell suspension medium into each of ten 100 mm petri plates. Tilt each plate to evenly distribute the medium to cover the bottom of the plate. Avoid the introduction of bubbles during plating. Incubate plates at 37°C in 5% CO<sub>2</sub> atmosphere. Do not disturb plates for 10 - 14 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 colonies. We recommend using a water-jacketed incubator. It is advisable to put the plates in a separate plastic container together*

For Research Use Only. Not For Therapeutic or Diagnostic Use.

| In North America            | In Europe                     | In Australia               | In Singapore            | STEMCELL Technologies |
|-----------------------------|-------------------------------|----------------------------|-------------------------|-----------------------|
| Toll-Free T. 1.800.667.0322 | Toll-Free T. 00.800.7836.2355 | Toll-Free T. 1.800.060.350 | T. 65.972.66660         | Version 2.2.0         |
| Toll-Free F. 1.800.567.2899 | Toll-Free F. 00.800.7836.2300 | F. +61 (03)9338.4320       | E. info.sg@stemcell.com | June 2009             |
| T. 1.604.877.0713           | T. +33 (0)4.76.04.75.30       | E. info.aus@stemcell.com   |                         | Catalog #28411        |
| F. 1.604.877.0704           | F. +33 (0)4.76.18.99.63       |                            |                         |                       |
| E. info@stemcell.com        | E. info.eu@stemcell.com       |                            |                         |                       |
| E. orders@stemcell.com      |                               |                            |                         |                       |

with a 100 mm petri dish containing 10 mL sterile distilled water (no lid!) to maintain moisture content in methylcellulose cultures. 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 hazy colonies.

## 2.6 Harvest

- 10 - 14 days after cells are plated in Medium D, examine the plates for the presence of colonies visible to the naked eye. A typical fusion will produce 1000 or more colonies over the ten plates. Remove isolated colonies (usually 500 - 1000 colonies are harvested) from the plates using a pipettor set to 10  $\mu$ L and sterile pipette tips. Pipette each clone into an individual well of a 96-well tissue culture plate containing 200  $\mu$ L of ClonaCell<sup>®</sup>-HY Growth Medium (Medium E). With the pipettor set at 150  $\mu$ L, pipette the entire contents of the well up and down several times to resuspend the colony. The colony does not need to be resuspended into a perfect single-cell suspension but should be dispersed sufficiently to get good growth. The resuspension of the colonies may be performed using a multi-channel pipettor after all selected colonies have been transferred to 96-wells. Ensure a new sterile tip is used for each clone to maintain clonality of the colony. Incubate the plates at 37°C in 5% CO<sub>2</sub> for 1 - 4 days without feeding.

*By the fourth day, 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 media that turns yellow sooner than 4 days. It is a good idea to 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.*

- Transfer 150  $\mu$ L 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.).
- Add 150  $\mu$ L of fresh Medium E to every well of the original hybridoma containing plates.
- Gently resuspend the hybridomas that showed a positive response in Step 2. Transfer 100  $\mu$ L of cells to each of 2 wells of a 24-well plate, containing 1 mL of Medium E.
- When cells have grown to a suitable density (approximately 4 x 10<sup>5</sup> cells/mL), freeze the cells (see Appendix IV, Section 4.4.1) from one well and expand the remaining positive clones in a T-25 cm<sup>2</sup> tissue culture flask containing 5 mL of Medium A and 5 mL of Medium E. This step adapts the cells to growth in Medium A. In addition, keep a sample of cells in Medium E, in case the cells don't adapt well to the 1:1 mixture.
- When cells have grown to a suitable density (approximately 4 x 10<sup>5</sup> cells/mL), transfer 5 - 10 mL of cell culture by pipette into 20 mL of Medium A in a T-75 cm<sup>2</sup>. Adjust the volume of cells to ensure the final cell concentration is between 1 x 10<sup>4</sup> - 5 x 10<sup>4</sup> cells/mL. Maintain expanded hybridomas in 100% Medium A at a concentration of 5 x 10<sup>4</sup> - 5 x 10<sup>5</sup> cells/mL. More aliquots of cells can be frozen at this point in order to secure the supply of the hybridoma.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

### In North America

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. info@stemcell.com  
E. orders@stemcell.com

### In Europe

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. info.eu@stemcell.com

### In Australia

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. info.aus@stemcell.com

### In Singapore

T. 65.972.66660  
E. info.sg@stemcell.com

### STEMCELL Technologies

Version 2.2.0  
June 2009  
Catalog #28411

## 3.0 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 500 to 1000 clones. 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 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 Medium A 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. Please refer to Section 2.2.
- Myeloma cells are contaminated with *mycoplasma*. *Mycoplasma* consumes 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 or an extended period of time 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 and harvesting the myeloma cells.
- Poor viability of the fused cells. Freshly fused cells are very 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.

### 3.2 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 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. Please refer to Appendix I for a suggested immunization schedule.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

| In North America            | In Europe                     | In Australia               | In Singapore            | STEMCELL Technologies |
|-----------------------------|-------------------------------|----------------------------|-------------------------|-----------------------|
| Toll-Free T. 1.800.667.0322 | Toll-Free T. 00.800.7836.2355 | Toll-Free T. 1.800.060.350 | T. 65.972.66660         | Version 2.2.0         |
| Toll-Free F. 1.800.567.2899 | Toll-Free F. 00.800.7836.2300 | F. +61 (03)9338.4320       | E. info.sg@stemcell.com | June 2009             |
| T. 1.604.877.0713           | T. +33 (0)4.76.04.75.30       | E. info.aus@stemcell.com   |                         | Catalog #28411        |
| F. 1.604.877.0704           | F. +33 (0)4.76.18.99.63       |                            |                         |                       |
| E. info@stemcell.com        | E. info.eu@stemcell.com       |                            |                         |                       |
| E. orders@stemcell.com      |                               |                            |                         |                       |

### 3.3 Hazy or Runny Colonies

- Methylcellulose is a viscous solution. Disturbing the dishes before Day 10 will break apart small forming colonies and cause them to appear hazy or runny. (Do not touch dishes for the first 10 days, even to sneak a peek at them).
- Low viscosity of Medium D due to improper thawing will result in hazy or runny colonies. It is not recommended to thaw Medium D 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 medium's viscosity.

### 3.4 Other

- If colonies in Medium D (Section 2.6) are not well distributed due to low viscosity of the medium, or if the colonies are hazy or runny, or if the cell density is too high, plucking individual colonies might be difficult. In this case we recommend recloning positive hybridomas once the cells have been established in Medium E (Section 2.6). Follow the protocol for Recloning in ClonaCell®-HY in Appendix IV, Section 4.4.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

#### In North America

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. [info@stemcell.com](mailto:info@stemcell.com)  
E. [orders@stemcell.com](mailto:orders@stemcell.com)

#### In Europe

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. [info.eu@stemcell.com](mailto:info.eu@stemcell.com)

#### In Australia

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. [info.aus@stemcell.com](mailto:info.aus@stemcell.com)

#### In Singapore

T. 65.972.66660  
E. [info.sg@stemcell.com](mailto:info.sg@stemcell.com)

#### STEMCELL Technologies

Version 2.2.0  
June 2009  
Catalog #28411

## 4.0 Appendix

### 4.1 Appendix I: Immunization of BALB/c Mice

The mice must be immunized with antigen 6 - 10 weeks before fusion to allow them to develop a robust immune response before generating hybridomas. This section provides an example of a typical injection schedule for immunizing Balb/c mice prior to the fusion. This is a suggested injection schedule only and the actual timing may vary depending on the antigen used for the immunization as well as other factors.<sup>9</sup> It is desirable to immunize mice with a pure antigen, as this simplifies the screening of hybridomas. However, complex antigenic mixtures can be used.

1. Collect a sample of serum or plasma prior to immunization to use as a baseline control for antibody screening. Bleed the mice by cutting approximately 1 - 2 mm off the tip of the tail, collect 100 - 200  $\mu$ L of blood in a heparin coated capillary tube and prepare plasma. Save this serum by adding 0.1% sodium azide and store at -20°C. Thaw just prior to use.
2. A typical immunization schedule is as follows: Inject 2 - 4 adult BALB/c mice with antigen. Typically 20 - 100  $\mu$ g of purified antigen or 100 - 200  $\mu$ g of antigen mixture is injected intraperitoneally in a total volume of 200  $\mu$ L (i.e. 200  $\mu$ L of a 1:1 emulsion of antigen in saline or adjuvant).
3. Preparation of a stable emulsion is critical to generate a strong immune response.
4. Repeat the injection 14 - 30 days later.
5. 10 - 14 days after the second injection, collect 100 - 200  $\mu$ L of blood by cutting 1 - 2 mm from the tip of the tail. Collect blood droplets in a heparin-coated capillary tube. Prepare plasma from the blood sample and measure antibody levels in ELISA, immunofluorescence, flow cytometry, immunoblotting, etc.
6. Be sure to compare with the pre-immune serum from the same animal.
7. Select the mouse with the highest antibody titres for further boosting with antigen. Continue to give injections at 2 week intervals until a good titre of antibody is obtained.
8. 1 - 4 days before the day of the fusion, depending on factors such as route of immunization, boost the selected BALB/c mouse intravenously via the tail vein. Usually antigen is dissolved/suspended in saline and a maximum of 200  $\mu$ L are injected. Prepare to fuse spleen cells 1 - 4 days later.
9. It is recommended to prewarm the mouse with a heat lamp and apply a topical anesthetic to tail prior to antigen injection.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

| In North America   | In Europe   | In Australia   | In Singapore                               | STEMCELL Technologies                        |
|--|---|--|--|--|
| Toll-Free T. 1.800.667.0322<br>Toll-Free F. 1.800.567.2899<br>T. 1.604.877.0713<br>F. 1.604.877.0704<br>E. info@stemcell.com<br>E. orders@stemcell.com | Toll-Free T. 00.800.7836.2355<br>Toll-Free F. 00.800.7836.2300<br>T. +33 (0)4.76.04.75.30<br>F. +33 (0)4.76.18.99.63<br>E. info.eu@stemcell.com | Toll-Free T. 1.800.060.350<br>F. +61 (03)9338.4320<br>E. info.aus@stemcell.com | T. 65.972.66660<br>E. info.sg@stemcell.com | Version 2.2.0<br>June 2009<br>Catalog #28411 |

## 4.2 Appendix II: Preparation of the Splenocyte Suspension

1. Sacrifice an immunized mouse according to procedures recommended by your institution and wash the fur with 95% ethanol. Clip fur and pull back to expose chest.
2. Remove spleen and place in a sterile petri dish containing 5 mL of Medium A. Trim off any large pieces of fatty tissue.
3. Disaggregate the spleen into a single cell suspension. A suggested method of disaggregation is to transfer the spleen to a screen placed on top of a 50 mL conical centrifuge tube, and use the plunger of a 3 mL syringe to grind the cells out of the spleen. Rinse the screen with Medium B to assist the cells through the screen. Only the spleen membrane should remain on the screen. Gently pipette the cells up and down to disrupt clumps, but try not to cause the solution to foam.

## 4.3 Appendix III: Alternative 96-well Plate Format for the Selection and Cloning of Hybridomas in ClonaCell®-HY Medium D

This method is an alternative to the standard cloning and selection method described above (Section 2.5) 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 10 cm culture dishes. Hybridomas develop in the semi-solid layer as discrete colonies and secrete antibodies into the surrounding medium. Liquid medium 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 below. 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. Refer to Figure 2 for an overview of the procedure.

Follow the procedure as described in Section 2.1 to 2.4.

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 2.4.
3. Incubate the fusion products for 16 – 24 hours in recovery ClonaCell®-HY Medium C at 37°C in a humidified, 5% CO<sub>2</sub> incubator.
4. On the day after the fusion, vigorously shake the thawed ClonaCell®-HY Medium D to mix the contents of the bottle and warm to room temperature.
5. Determine the optimal number of cells to plate per well to arrive at one colony per well. We recommend a range of 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 recovery medium (ClonaCell®-HY Medium C) for a total volume of 10 mL. It is critical not to 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.*
6. Combine 10 mL of fused cell suspension with 90 mL of ClonaCell-HY Medium D. Gently invert the bottle 6X to mix thoroughly and let sit for 15 minutes to allow the bubbles to surface.
7. Using either a multi-channel pipettor and sterile wide bore pipette tips (Axygen, Catalog# T-205-WB-C-R-S or equivalent) or a Distriman repeat pipettor (Gilson, Catalog# F164001) and DistriTip (Gilson, Catalog#

For Research Use Only. Not For Therapeutic or Diagnostic Use.

### In North America

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. info@stemcell.com  
E. orders@stemcell.com

### In Europe

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. info.eu@stemcell.com

### In Australia

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. info.aus@stemcell.com

### In Singapore

T. 65.972.66660  
E. info.sg@stemcell.com

### STEMCELL Technologies

Version 2.2.0  
June 2009  
Catalog #28411

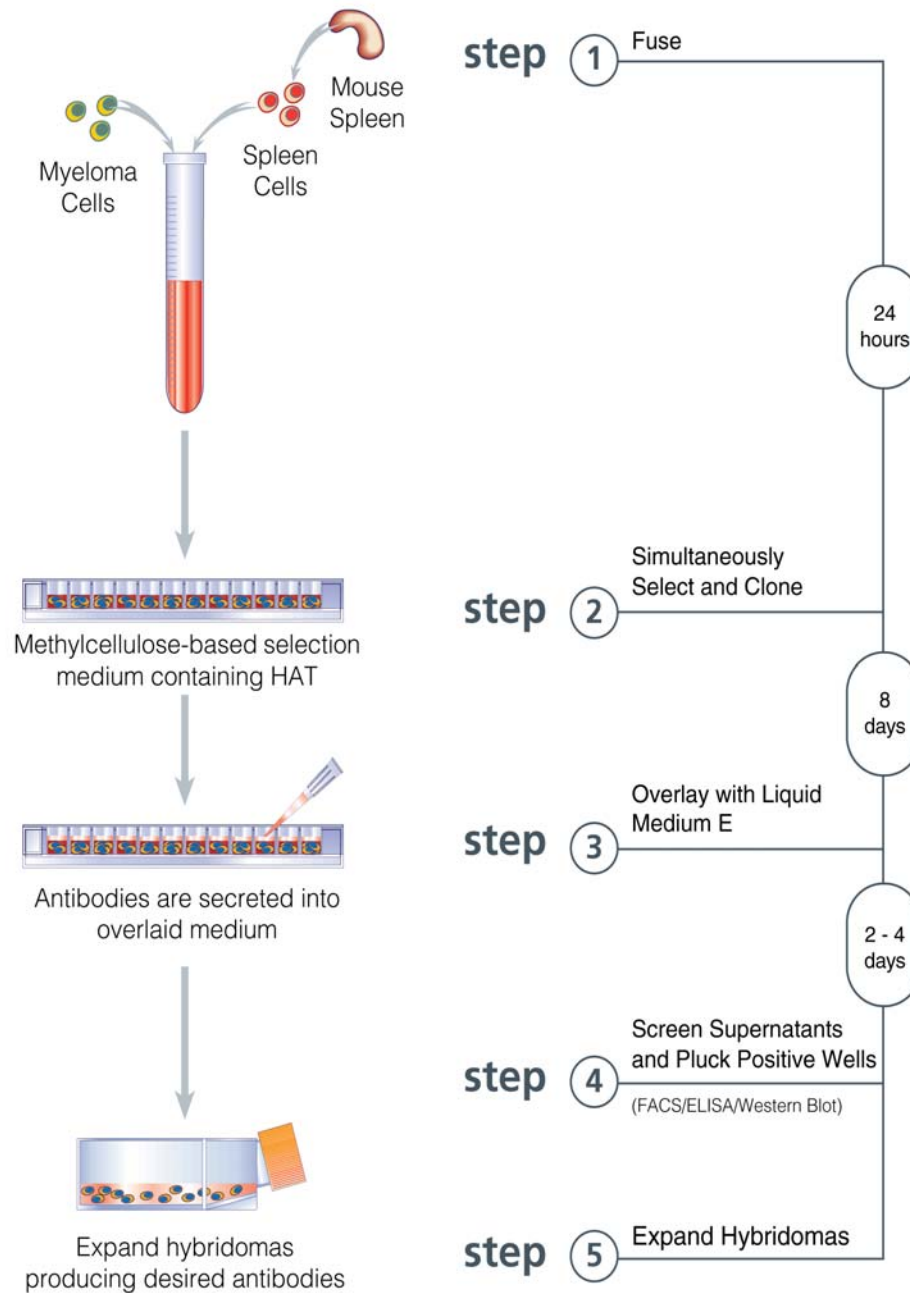
F164140), dispense 60 – 80 µL of ClonaCell®-HY Medium D into each well of 96-well plates. This will yield between 12-16 plates depending on the volume plated. ClonaCell®-HY Medium D is a viscous solution and therefore difficult to pipette accurately. However, it is not critical to dispense exactly the same volume into each well.

8. Incubate the plates at 37°C in a humidified, 5% CO<sub>2</sub> incubator. The incubator should be well humidified to prevent excessive evaporation. If desired, the plates may be placed inside a plastic container (e.g., Corning square bioassay 245mm X 245mm dish [Cat# 431111]) along with an open Petri dish containing distilled water. If using a regular storage container make sure that the lid is fit on loosely to facilitate gas exchange.
9. Following 8 days of undisturbed incubation, examine wells for the presence of colonies and gently overlay 150 µL of pre-warmed ClonaCell®-HY Medium E onto the semi-solid medium of each well containing colonies. Alternatively, all wells may be overlaid with 150 µL of pre-warmed ClonaCell®-HY 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 in a humidified, 5% CO<sub>2</sub> incubator. The overlay incubation time may be increased further to ensure the detection of low expressing hybridomas.
11. Carefully remove 100 µL of the overlaid ClonaCell®-HY 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 etc.).
12. The contents of wells that tested positive for antibodies against the antigen of interest should be gently resuspended and transferred to wells of a 24-well plate containing 1 mL of ClonaCell®-HY 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 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 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 harvested from wells containing only a single hybridoma or for individual hybridomas harvested from wells containing more than 1 colony as these hybridomas should already be monoclonal. However, it may still be useful to reclone these hybridomas to select for stable high-producing subclones.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

| In North America   | In Europe   | In Australia   | In Singapore                               | STEMCELL Technologies                        |
|--|---|--|--|--|
| Toll-Free T. 1.800.667.0322<br>Toll-Free F. 1.800.567.2899<br>T. 1.604.877.0713<br>F. 1.604.877.0704<br>E. info@stemcell.com<br>E. orders@stemcell.com | Toll-Free T. 00.800.7836.2355<br>Toll-Free F. 00.800.7836.2300<br>T. +33 (0)4.76.04.75.30<br>F. +33 (0)4.76.18.99.63<br>E. info.eu@stemcell.com | Toll-Free T. 1.800.060.350<br>F. +61 (03)9338.4320<br>E. info.aus@stemcell.com | T. 65.972.66660<br>E. info.sg@stemcell.com | Version 2.2.0<br>June 2009<br>Catalog #28411 |

**Figure 2. ClonaCell®-HY 96-Well Procedure**



For Research Use Only. Not For Therapeutic or Diagnostic Use.

**In North America**

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. info@stemcell.com  
E. orders@stemcell.com

**In Europe**

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. info.eu@stemcell.com

**In Australia**

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. info.aus@stemcell.com

**In Singapore**

T. 65.972.66660  
E. info.sg@stemcell.com

**STEMCELL Technologies**

Version 2.2.0  
June 2009  
Catalog #28411

## 4.4 Appendix IV: Recloning in ClonaCell®-HY

Recloning is recommended if a culture is suspected to be not monoclonal (i.e. if the cell density in the plates was high and it is possible that cells of 2 or more colonies have been harvested).

Recloning is also recommended for hybridomas that have been in continuous culture for an extended period, in particular if antibody production has declined and selection of high antibody secreting subcultures is desired. Reclone hybridomas in ClonaCell®-HY Medium D (Catalog #03804) according to the following protocol:

1. Culture the hybridomas in 10 mL of Medium E at a maximum cell density of  $2 \times 10^5$  cells/mL. Prepare a cell suspension at a density of 100 cells/mL in Medium A.
2. In a 15 mL conical tube, mix 9 mL of Medium D and 1 mL of hybridoma cell suspension (100 cells). Let sit at 37°C for 15 minutes to allow air bubbles to rise.
3. Plate out the suspension in one petri plates as indicated in Section 2.5, Step 4. 10 - 14 days later, examine the plates for the presence of colonies visible to the naked eye. Assuming a plating efficiency of 50 - 80%, there will be 50 - 80 colonies in the plate.
4. Remove 15 - 20 colonies from the plate using a pipettor set to 10  $\mu$ L and sterile pipette tips. Pipette each clone into an individual well of a 96-well tissue culture plate containing 200  $\mu$ L of Medium E. Incubate the plates at 37°C in 5% CO<sub>2</sub> for 1 - 4 days. Do not let cells overgrow.
5. Continue as indicated in Section 2.6, Steps 2 to 6.

## 4.5 Appendix V: Freezing and Thawing Cells

### 4.5.1 Freezing Hybridomas

1. Cryopreserve cells at a concentration of  $2 \times 10^6$  cells per vial.
2. Label the required number of sterile 2 mL cryovials (1.8 mL capacity).
3. Prepare a 20% DMSO solution in Fetal Bovine Serum (FBS). Place FBS in a 50 mL conical tube and cool on ice. Slowly add the appropriate volume of DMSO and mix well. Filter sterilize solution using a 0.2  $\mu$ m filter and keep on ice.
4. Harvest cells and resuspend in cold FBS at twice the desired final cell concentration (e.g. suspend at  $4 \times 10^6$  cells/mL for cells cryopreserved at  $2 \times 10^6$  cells per cryovial).
5. Slowly add the 20% DMSO in FBS solution at a ratio of 1:1 to the tube containing the cells. Continue to mix during the addition. Transfer 1 mL of cells in freezing medium to each cryovial. *The final cell suspension will be in 90% FBS: 10% DMSO.*
6. Place cryovials immediately into freezing containers. To ensure good viability and cell recovery, do not let cells sit in freezing media at room temperature. Keep on ice and transfer within 15 minutes to the freezing container. Handle freezing container according to manufacturer's instructions.
7. Place container in -70°C or -135°C freezer overnight.
8. Next day, remove frozen vials from the freezing container and store in liquid nitrogen.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

| In North America            | In Europe                     | In Australia               | In Singapore            | STEMCELL Technologies |
|-----------------------------|-------------------------------|----------------------------|-------------------------|-----------------------|
| Toll-Free T. 1.800.667.0322 | Toll-Free T. 00.800.7836.2355 | Toll-Free T. 1.800.060.350 | T. 65.972.66660         | Version 2.2.0         |
| Toll-Free F. 1.800.567.2899 | Toll-Free F. 00.800.7836.2300 | F. +61 (03)9338.4320       | E. info.sg@stemcell.com | June 2009             |
| T. 1.604.877.0713           | T. +33 (0)4.76.04.75.30       | E. info.aus@stemcell.com   |                         | Catalog #28411        |
| F. 1.604.877.0704           | F. +33 (0)4.76.18.99.63       |                            |                         |                       |
| E. info@stemcell.com        | E. info.eu@stemcell.com       |                            |                         |                       |
| E. orders@stemcell.com      |                               |                            |                         |                       |

#### 4.5.2 Thawing and Culturing of Cells (Parental Myeloma Cells, Hybridomas)

1. Store cells in liquid nitrogen until ready to use.
2. Place 10 mL of pre-warmed Medium A into a sterile 15 mL tube.
3. Thaw cells quickly by agitating the vial in a 37°C water bath.
4. Draw up the cell suspension in a 2 mL pipette and place in a 15 mL tube. Add 10 mL of culture medium dropwise. Centrifuge at 400 x g (~1350 rpm) for 10 minutes. *This wash step is required to remove DMSO.*
5. Discard the supernatant and resuspend the cells in 1 - 2 mL of culture media.
6. Determine the viable cell concentration using a hemacytometer and calculate the volume of cells necessary to seed at a cell density of  $\sim 5 \times 10^4$  viable cells/mL.
7. Use the appropriate culture media to have a final volume of 10 mL/T-25 cm<sup>2</sup> flask or 30 mL/T-75 cm<sup>2</sup> flask.
8. Place the flask in a 37°C incubator with 5% CO<sub>2</sub> in air with 95% humidity.
9. Grow cells to the cell density suggested by the protocol and passage cells every 2 - 4 days.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

##### In North America

Toll-Free T. 1.800.667.0322  
Toll-Free F. 1.800.567.2899  
T. 1.604.877.0713  
F. 1.604.877.0704  
E. [info@stemcell.com](mailto:info@stemcell.com)  
E. [orders@stemcell.com](mailto:orders@stemcell.com)

##### In Europe

Toll-Free T. 00.800.7836.2355  
Toll-Free F. 00.800.7836.2300  
T. +33 (0)4.76.04.75.30  
F. +33 (0)4.76.18.99.63  
E. [info.eu@stemcell.com](mailto:info.eu@stemcell.com)

##### In Australia

Toll-Free T. 1.800.060.350  
F. +61 (03)9338.4320  
E. [info.aus@stemcell.com](mailto:info.aus@stemcell.com)

##### In Singapore

T. 65.972.66660  
E. [info.sg@stemcell.com](mailto:info.sg@stemcell.com)

##### STEMCELL Technologies

Version 2.2.0  
June 2009  
Catalog #28411

## 5.0 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. Melchers F, Potter M, Warner NL: *Lymphocyte hybridomas. Second workshop on "functional properties of tumors of T and B lymphocytes"*. Preface. *Current Topics in Microbiology & Immunology* 81: IX-XXIII, 1978
4. 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.
5. Goding JW: Antibody production by hybridomas. [Review]. *J Immunol Methods* 39: 285-308, 1980.
6. Sharon J, Morrison SL, Kabat EA: Detection of specific hybridoma clones by replica immunoadsorption of their secreted antibodies. *Proc Natl Acad Sci (USA)* 76: 1420-4, 1979.
7. Pearson TW, Pinder M, Roelants G, Kar S, Lundin L, Mayor-Whitney KS, Hewett RS: Methods for derivation and detection of anti-parasite monoclonal antibodies. *J Immunol Methods* 34: 141-154, 1980.
8. Kennett RH, McKearn TJ, Bechtol KB: *Monoclonal antibodies*. Plenum Press, New York, 1980.
9. Coligan JE: *Current Protocols in Immunology*, Unit 2.5: Production of Monoclonal Antibodies. John Wiley & Sons, Inc., USA, 2006.

For Research Use Only. Not For Therapeutic or Diagnostic Use.

| In North America   | In Europe   | In Australia   | In Singapore                               | STEMCELL Technologies                        |
|--|---|--|--|--|
| Toll-Free T. 1.800.667.0322<br>Toll-Free F. 1.800.567.2899<br>T. 1.604.877.0713<br>F. 1.604.877.0704<br>E. info@stemcell.com<br>E. orders@stemcell.com | Toll-Free T. 00.800.7836.2355<br>Toll-Free F. 00.800.7836.2300<br>T. +33 (0)4.76.04.75.30<br>F. +33 (0)4.76.18.99.63<br>E. info.eu@stemcell.com | Toll-Free T. 1.800.060.350<br>F. +61 (03)9338.4320<br>E. info.aus@stemcell.com | T. 65.972.66660<br>E. info.sg@stemcell.com | Version 2.2.0<br>June 2009<br>Catalog #28411 |





THE CELL EXPERTS™ | [WWW.STEMCELL.COM](http://WWW.STEMCELL.COM)

TOLL-FREE PHONE 1 800 667 0322 • PHONE 1 604 877 0713

TOLL-FREE FAX 1 800 567 2899 • FAX 1 604 877 0704

[ORDERS@STEMCELL.COM](mailto:ORDERS@STEMCELL.COM) • [INFO@STEMCELL.COM](mailto:INFO@STEMCELL.COM)

FOR FULL CONTACT DETAILS WORLDWIDE VISIT OUR WEBSITE

FOR RESEARCH USE ONLY. NOT FOR THERAPEUTIC OR DIAGNOSTIC USE.      MANUAL CATALOG #28411      VERSION 2.2.0