TECHNICAL MANUAL
VERSION 1.0.0

ClonaCell®-HY Liquid HAT Hybridoma Selection

STEMCELL TECHNOLOGIES

For Research Use Only

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
E-mail: info@stemcell.com
www.stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.79.15.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies
Version 1.0.0
January 2007
29044

Table of Contents

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies

Version 1.0.0
February 2007
Catalog #29044

For Research Use Only

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies
Version 1.0.0
February 2007
Catalog #29044

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[1], for which work they were awarded the Nobel Prize in Physiology and Medicine in 1984. A variety of methods have since been used to fuse, grow, select, and clone hybridomas[2,3,4-7]. Traditionally the fusion products of spleen immune cells and myeloma cells are placed in culture flasks or wells with liquid selective medium containing Hypoxanthine, Aminopterin, and Thymidine (HAT) that promotes the survival and proliferation of hybridoma cells and the elimination of non-fused B cells and myeloma cells. Cultures containing expanding hybridoma cells are then screened for the presence of antibodies with the desired antigen specificity. Cells from cultures that are identified as positive for producing the desired antibody are then subcultured using a limiting dilution approach to ensure that a monoclonal antibody-producing cell line is obtained.

ClonaCell®-HY Liquid HAT Hybridoma Selection Medium is a DMEM-based formulation containing growth factors and medium supplements optimized to support the generation, selection, and growth of large numbers of hybridoma clones.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies

Version 1.0.0
February 2007
Catalog #29044

1.2 Description

ClonaCell®-HY Liquid HAT Hybridoma Selection Medium is a special formulation of DMEM-based medium containing growth factors, B cell stimulators and medium supplements optimized for the outgrowth of hybridomas.

The advantages of using the ClonaCell®-HY Liquid HAT Medium together with other ClonaCell®-HY reagents over standard methods and other commercially available hybridoma selection media are described below:

- All the necessary reagents are available for all steps, from cell fusion to expansion of selected hybridomas.
- Reagents are pre-screened, reducing the time spent screening medium components and troubleshooting.
- All reagents are pre-mixed and require no additional supplements, reducing the amount of time spent preparing medium.
- All reagents have been optimized to give high plating efficiency ensuring maximum hybridoma yield.
- Frozen medium can be thawed the day before use, allowing more flexibility in scheduling fusion and screening experiments.
- The antibiotic gentamycin is included in the media to suppress growth of a broad range of potential microbial contaminants, including *Mycoplasma sp*.
- Includes growth factors that promote outgrowth of hybridomas, resulting in higher hybridoma yields with each fusion.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

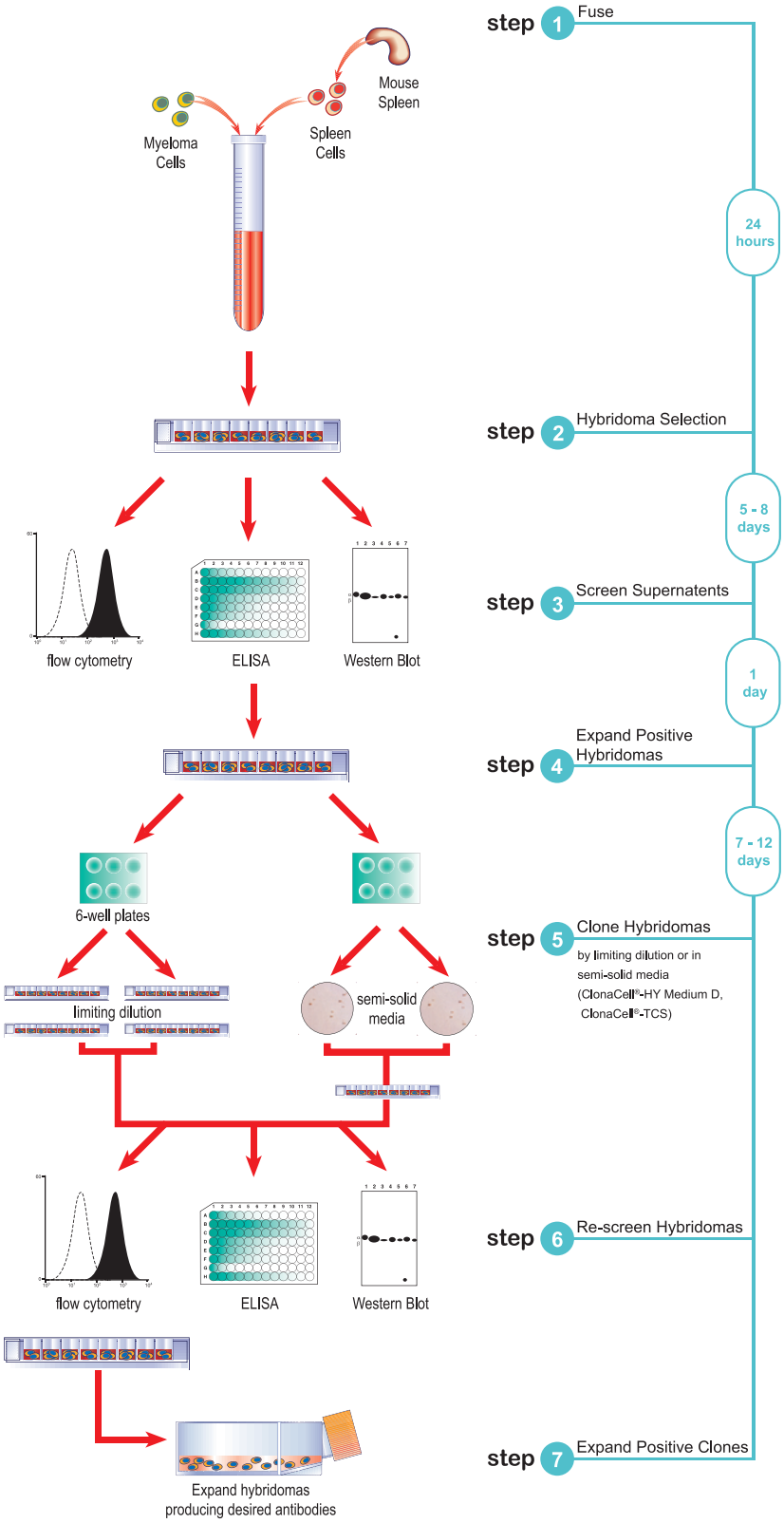
In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies

Version 1.0.0
February 2007
Catalog #29044

Figure 1. ClonaCell®-HY Liquid HAT Procedure Overview



For Research Use Only

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies

Version 1.0.0
February 2007
Catalog #29044

1.3 ClonaCell®-HY Products for Hybridoma Selection in ClonaCell®-HY Liquid HAT Medium:

Medium A	**ClonaCell®-HY Pre-Fusion Medium and Hybridoma Expansion Medium** Catalog #03801, 500 mL Contains Dulbecco's Modified Eagle's Medium (DMEM), pre-selected serum, gentamycin, and supplements.
Medium B	**ClonaCell®-HY Fusion Medium** Catalog #03802, 500 mL Contains DMEM and gentamycin.
Medium C	**ClonaCell®-HY Hybridoma Recovery Medium** Catalog #03803, 100 mL Contains DMEM, pre-selected serum, gentamycin, and supplements.
HAT Medium	**ClonaCell®-HY Liquid HAT Hybridoma Selection Medium** Catalog #03831, 500 mL Contains DMEM, pre-selected serum, HAT, gentamycin, and supplements.
Medium E	**ClonaCell®-HY Hybridoma Growth Medium** Catalog #03805, 500 mL Contains DMEM, pre-selected serum, HT, gentamycin, and supplements.
Polyethylene Glycol	**ClonaCell®-HY PEG Solution** Catalog #03806, 1.5 mL Contains 50% PEG solution pretested for cell fusion in DMEM.

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_2 in air
- Pipette-aid
- Hemacytometer
- Routine light microscope
- Inverted microscope
- 37°C water bath
- Liquid nitrogen tank and freezing head
- Freezing container (e.g. Nalgene, Catalog #5100); optional

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies
Version 1.0.0
February 2007
Catalog #29044

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
- 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^2 sterile tissue culture flask (Corning, Catalog #430639, or equivalent)
- T-75 cm^2 sterile tissue culture flask (BD, Catalog #353136, or equivalent)
- 96-well sterile tissue culture plates (Catalog #27136)
- 24-well sterile tissue culture treated plates (Corning, Catalog #003526, or equivalent)
- 10 cm petri dish (*optional: for 2.8 Cloning - Method A*)
- 96-well ELISA plates (Nunc, Catalog #537336, or equivalent)
- 12 mL syringe (*optional: for 2.8 Cloning - Method A*)
- Blunt-end 16 gauge needle (*Catalog #28110; optional: for 2.8 Cloning - Method A)*
- Forceps (2)
- Fine scissors
- Fine-mesh metal screen or disposable cell strainer (Catalog #27305)
- Multi-channel pipettor, 12-channel, 20 - 200 µL

Biologicals

- Myeloma Cell Line (e.g. SP2/0, X63Ag8.653)
- Primed mouse 1 - 4 days after final antigen boost

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies

Version 1.0.0
February 2007
Catalog #29044

2.0 Methods

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

2.1 Mouse Immunization

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

2.2 Myeloma Cells

The parental myeloma cells 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. 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 and X63Ag8.653) are widely available. Be sure to obtain a parental myeloma cell that has been proven to yield good stable hybridomas.

1. Thaw the parental myeloma cells (see Appendix IV in Section 4.4.2) and culture in ClonaCell®-HY Pre-Fusion Medium (Medium A) for at least 1 week to ensure that they are well adapted to this medium prior to fusion. Seed cells at a density of approximately 5 x 10^4 cells/mL and passage every 2 days. Suggested maximum cell density is 4 x 10^5 cells/mL.

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

   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.

3. Harvest the parental myeloma cells in a 50 mL conical centrifuge tube by centrifuging at room temperature (RT) or 37ºC, 300 x *g* (~1100 rpm) for 10 minutes. Wash 3 times by adding 30 mL of ClonaCell®-HY Fusion Medium (Medium B), centrifuging again as before, and removing the supernatant. Resuspend the cell pellet in 25 mL of Medium B.

   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.

4. Count live cells using a viability stain. Viability of parental myeloma cells should be greater than 95%.

5. Calculate the volume of cell suspension that contains 2 x 10^7 viable cells. Keep cells at RT or 37ºC until fusion (Section 2.4).

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies

Version 1.0.0
February 2007
Catalog #29044

2.3 Splenocytes

1. Disaggregate the spleen into a single cell suspension as described in Appendix II, Section 4.2. Wash the splenocytes 3 times in 30 mL of Medium B, centrifuging at 400 x *g* (~1350 rpm) at RT or 37ºC for 10 minutes each time and removing the supernatant by pipette. After the final wash resuspend the cells in 25 mL Medium B.

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.

2. Prepare a 1/10 dilution of cells in 3% acetic acid, e.g. by mixing 10 µL of the cell suspension with 90 µL of 3% Acetic Acid with Methylene Blue (Catalog #07060).

3. Count cells in this diluted sample using a hemacytometer. Calculate the volume of cell suspension that contains 1 x 10^8 cells.

2.4 Fusion

Prewarm PEG and media (Medium A, B, C) to 37ºC and prepare a 37ºC water bath if using Method A (below).

1. Add 2 x 10^7 parental myeloma cells and 1 x 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.

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

2. Fuse cells using 1 of the 2 methods outlined below:

Method A

3a. Break up the cell pellet obtained in Section 2.4 by gently tapping the bottom of the tube. The pellet should be disrupted for optimal fusion. Slowly add 1 mL of ClonaCell®-HY PEG Solution 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.

4a. Add 4 mL Medium B to the fusion mixture, continuously stirring as before, over a period of 4 minutes.

5a. Slowly add 10 mL Medium B. Incubate for 15 minutes in 37°C water bath.

6a. Slowly add 30 mL of Medium A and centrifuge the cells at 400 x *g* for 7 minutes. Discard the supernatant and wash cells with 40 mL of the Medium to ensure that all the PEG is removed.

7a. 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^2 tissue culture flask containing 40 mL of Medium C (total culture volume = 50 mL). Incubate for 16 - 24 hours at 37°C in 5% CO_2 atmosphere.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies

Version 1.0.0
February 2007
Catalog #29044

Method B

3b 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®-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 the 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.

4b Carefully add 5 mL of Medium B dropwise to the pellet while gently swirling the tube to resuspend the cells.

5b Slowly add 5 mL of ClonaCell®-HY Hybridoma Recovery Medium (Medium C) to the solution. Continue to swirl the tube.

6b Transfer the cell suspension to a T-75 cm^2 tissue culture flask containing 40 mL of Medium C (total culture volume = 50 mL). Incubate for 16 - 24 hours at 37°C in 5% CO_2 atmosphere.

There will still be clumps of cells at this point which will dissolve overnight. Be gentle with these cells.

2.5 Growth of Hybridomas

1. On the day of the fusion, place ClonaCell®-HY Liquid HAT Hybridoma Selection Medium at 2 - 8°C and thaw overnight. On the day after the fusion, invert the medium several times to mix contents well. As only 90 mL of the ClonaCell®-HY Liquid HAT Medium is required for this procedure, this medium can be aliquoted and frozen for later use. It is recommended to prepare the medium in 100 mL aliquots. Warm medium to 37°C before use.

2. Transfer the 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 by pipette. Resuspend the cells in ClonaCell®-HY Liquid HAT Medium to a total volume of 10 mL.

3. Transfer the 10 mL cell suspension into 90 mL of ClonaCell®-HY Liquid HAT Medium. Mix thoroughly by gently inverting the bottle.

4. Using a 12-channel pipettor, aseptically plate out 100 µL of cell suspension medium into each well of ten 96-well tissue culture plates. Incubate plates at 37°C in 5% CO_2 atmosphere. 5 days after fusion, feed the plates with 100 µL/well of ClonaCell®-HY Hybridoma Growth Medium (Medium E). Continue feeding as required.

2.6 Screening

NOTE: It is important to have a screening assay well established prior to performing the fusion.

1. 7 - 10 days after the cells are plated in ClonaCell®-HY Liquid HAT Medium, inspect each well for hybridoma growth. Aseptically transfer 150 µL of the supernatant from each hybridoma well to a separate well on a new 96-well plate and analyze by an assay system appropriate for the antigen involved (e.g. ELISA, flow cytometry, or Western Blot).

If a large number of positive wells are obtained and/or the screening cannot be completed quickly, it may be desirable to make and freeze a replica plate in order to minimize the possibility of losing useful hybridomas due to overgrowth.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies
Version 1.0.0
February 2007
Catalog #29044

2. Add 150 µL of fresh Medium E to every well of the original hybridoma containing plates.

3. Continue feeding the wells every 2 days with Medium E by removing 100 µL of supernatant and adding 100 µL of fresh prewarmed Medium E.

   Do not let the wells overgrow or the media turn yellow. ClonaCell®-HY Medium E does not contain aminopterin, so feeding the cells also serves to dilute the aminopterin from the media, which allows for the cells to be transfered to ClonaCell®-HY Medium A or other growth medium.

2.7 Expansion and Freezing

1. Gently resuspend the hybridomas that showed a positive response in Section 2.6. Transfer 100 µL each to 2 wells of a 24-well plate, containing 1 mL of Medium E.

2. When cells have grown to a suitable density (approximately 4 x 10^5 cells/mL), freeze the cells (see Appendix IV, Section 4.4.1) from 1 well and reclone the remaining positive clones (see Section 2.8).

Note: *The cells are not monoclonal at this stage. Freezing is performed to secure the hybridoma in the event that the expansion and recloning of the cells do not work.*

2.8 Cloning

Clone hybridomas by either of the two methods described below.

Method A: Cloning in ClonaCell®-HY or ClonaCell®-TCS semi-solid medium

If the hybridoma cells have been weaned off aminopterin (i.e. the cells have been fed several times with ClonaCell®-HY Medium E), it may be preferable to reclone in the methycellulose-based ClonaCell®-TCS medium (Catalog #03814) as this medium does not contain aminopterin. Otherwise, methylcellulose-based ClonaCell®-HY Medium D (Catalog #03804), which contains aminopterin, can be used for recloning.

Note: *This method requires ClonaCell® -HY Medium D or ClonaCell® -TCS which is not included in this kit and must be ordered separately.*

1a. The day before recloning, feed the well to be recloned with fresh Medium E by removing 100 µL of supernatant and adding 100 µL of fresh medium.

2a. Resuspend the cells in the well to be recloned by pipetting up and down several times.

3a. Measure cell viability using Trypan Blue. Measure the cell concentration using a hemacytometer.

   Trypan Blue can underestimate viability since some cells that are no longer capable of reproducing will still be able to exclude trypan. When counting hybridomas, exclude those cells that appear mis-shaped, even if they are not stained blue.

4a. Add 9 mL of ClonaCell®-HY Medium D or ClonaCell®-TCS to a 15 mL sterile round-bottom tube.

5a. Add 100 - 200 viable cells and liquid medium (e.g. ClonaCell®-HY Medium E, ClonaCell®-HY Medium A, or DMEM) to the tube prepared in Step 4a. The final volume at this point should be 10 mL. Mix by pipetting up and down using a 12 mL syringe and blunt end needle.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies
Version 1.0.0
February 2007
Catalog #29044

6a. Using a blunt end syringe, aseptically transfer the cell suspension medium into a sterile 10 cm petri dish. Tilt the plate to level mixture and try not to introduce bubbles. Place the dish in an incubator operating at 37°C with 5% CO_2 for 10 - 14 days.

Do not disrupt the dish for the first 10 days, even to examine cells under a microscope. Doing so will result in runny colonies.

7a. After 10 - 14 days, harvest cloned colonies using a P20 pipettor set to 10 µL. Transfer the harvested colony to one well of a 96-well plate containing 200 µL of Medium E.

8a. Incubate the plates in a humidified incubator at 37°C, with 5% CO_2 for 1 - 4 days without feeding.

9a. Transfer 150 µL of each hybridoma supernatant to a separate well on a new 96-well plate and analyze by an assay system appropriate for the antigen involved (e.g. ELISA, flow cytometry, Westen Blot, etc.)

10a. Add 150 µL of fresh Medium E to every well of the original hybridoma containing plates.

11a. Gently resuspend the hybridomas that showed a positive response in step 9a. Transfer 100 µL each to 2 wells of a 24-well plate, containing 1 mL of Medium E.

12a. When cells have grown to a suitable density (approximately 4 x 10^5 cells/mL), freeze the cells (see Appendix IV, Section 4.4.1) from 1 well and expand the remaining positive clones in a T-25 cm^2 tissue-culture flask containing 5 mL of Medium A and 5 mL of Medium E.

13a. Keep a sample of cells in Medium E, in case they don't adapt well to the 50/50 mixture.

14a. When cells have grown to a suitable density (approximately 4 x 10^5 cells/mL) in the T-25 cm^2 flask, transfer 5 - 10 mL of the cell culture into 30 mL of Medium A in a T-75 cm^2 flask. Adjust the volume of cells to ensure the final cell concentration is 1 - 5 x 10^4 cells/mL.

15a. Maintain expanded hybridomas in 100% Medium A at a concentration of 5 x 10^4 - 5 x 10^5 cells/mL. More aliquots of cells can be frozen at this point in order to secure the supply of the hybridoma.

Method B: Cloning by limiting dilution

Round bottom 96-well plates are recommended in this protocol (see Step 2.8.8b). Cells roll to the bottom of the well and can be directly identified by microscope to determine if there is more than 1 cell per well; this is not possible with a flat bottom 96-well plate. This will allow you eliminate wells that receive more than 1 cell during plating.

1b. The day before recloning, perform a half media change (100 µL) with fresh Medium E.

2b. The day of recloning, resuspend the cells in the well to be recloned by pipetting up and down several times.

3b. Measure cell viability using Trypan Blue. Measure cell concentration using a hemacytometer.

Trypan Blue can underestimate viability, since some cells that are no longer capable of replication will still be able to exclude trypan. When counting hybridomas, exclude those cells that appear mis-shaped, even if they are not stained blue.

4b. Add 10 mL of Medium E to a 15 mL sterile centrifuge tube.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies
Version 1.0.0
February 2007
Catalog #29044

5b. Add a total of 80 viable cells to the tube prepared in Step 3b. This may require a serial dilution of the cells to avoid pipetting very small volumes of cells. Do not add more than 80 viable cells at this point. Mix by inverting the tube several times.

6b. Transfer the diluted cells to a reservoir, and then with a 12-channel pipettor transfer 100 µL of the cell preparation to each well of a 96-well plate.

7b. Place plates in a humidified incubator at 37°C containing 5% CO_2.

8b. *Optional*: After 1 hour, remove the plates from the incubator and examine each using an inverted microscope. It is often possible at this stage to identify wells that contain more than 1 cell. These cells are not monoclonal, so they should be excluded from subsequent expansion. Place an X on the cover of the dish over those wells that have received more than 1 cell.

9b. After 7 - 14 days, approximately 50 - 65% of the wells should have hybridomas growing in them. Assay the supernatant to confirm that they are still producing the antibody of interest, and expand the cells (see 2.8 Method A, Steps 11a - 15a) for antibody production or freezing.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies

Version 1.0.0
February 2007
Catalog #29044

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 of 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 broken up prior to the addition of PEG.
- The cells were exposed to PEG for too long resulting in cell death.

Possible causes of a low hybridoma viability and cloning efficiency:

- Poor growth or low viability of myeloma cells prior to fusion. Poor growth may occur if myeloma cells have not been sufficiently adapted to Medium A and are used too soon after switching to Medium A or after initiating the culture from cryopreserved cells. Low hybridoma 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* consume thymidine, which can result in low numbers of recovered hybridomas during the HAT selection.
- Poor viability of spleen cells prior to fusion. There may be several causes for 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 hr) 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 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.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies

Version 1.0.0
February 2007
Catalog #29044

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. These include:

- Too low a dose or low immunogenicity of the antigen. The optimal dose and immunogenicity are 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 1 for a suggested immunization schedule.

3.3 Low Cloning Efficiency of Positive Hybridomas

- Poor viability or low growth rate of the hybridoma at the time of cloning, as well as inherent genetic instability of the cells, may result in very few growing hybridomas after cloning. If positive clones can still be identified it is recommended to reclone this hybridoma to ascertain that it is monoclonal and stable. If you are uncertain about cell viability or quality of the hybridoma cells at the time of cloning it may be useful to plate cells at different densities. For example, if cloning in liquid medium an average of 5 cells per well could be deposited into the first 2 rows of wells of a 96-well plate, 1 cell per well in the next 3 rows and 0.5 cell per well of the last 3 rows.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies
Version 1.0.0
February 2007
Catalog #29044

4.0 Appendix

4.1 Appendix I: Immunization of BALB/c Mice

The mice must be immunized with antigen 6 - 10 weeks before the fusion to allow them to develop a robust immune response before the generation of 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[8]. 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. It is recommended to collect a sample of serum or plasma prior to immunization to use as a baseline control for antibody screening. Bleed the mice by clipping approximately 1 - 2 mm off the tip of the tail, collect 100 - 200 µL of blood and prepare serum. The easiest way to collect blood from a tail bleed is by using a capillary tube. Save the serum or plasma. Add 0.1% sodium azide and store at -20°C.

2. A typical immunization schedule is as follows:

   Inject 2 - 4 adult BALB/c mice with antigen. Typically 20 - 100 µg of purified antigen or 100 - 200 µg of antigen mixture is injected intraperitoneally in a total volume of 200 µL (i.e. 200 µL of a 1:1 emulsion of antigen in saline or adjuvant).

   Note: *Preparation of a stable emulsion is critical to generate a strong immune response.*

3. Repeat the injection 14 - 30 days later.

4. 10 - 14 days later, take 100 - 200 µL of blood by cutting 1 - 2 mm from the tip of the tail and collecting it into a capillary tube. Prepare serum from the blood sample and titrate in ELISA, immunofluorescence, flow cytometry, immunoblotting, etc.

   Be sure to compare with the pre-immune serum from the same animal.

5. 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.

6. 1 - 4 days before the day of the fusion, depending on factors such as route of immunization, and not earlier than 4 days in advance, boost the selected BALB/c mouse intravenously via the tail vein with 10 - 50 µg antigen. Usually antigen is dissolved/suspended in saline and a maximum of 200 µL are injected. Prepare to fuse spleen cells 3 - 4 days later.

   Note: *It is recommended to prewarm the mouse with a heat lamp and apply a topical anesthetic to the tail prior to antigen injection.*

4.2 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 Medium A. Trim off any large pieces of fatty tissue.

3. Disaggregate the spleen into a single cell suspension. 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 centrifuge tube, and use the plunger of a 3 mL syringe to grind the cells through the screen. Rinse the screen with Medium B to help all of the cells go through the screen. All that should be left in the screen is the spleen membrane. Gently pipette the cells up and down to disrupt clumps. Try not to cause the solution to foam.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies
Version 1.0.0
February 2007
Catalog #29044

4.3 Appendix III: Recloning in ClonaCell®-HY Medium D or ClonaCell®-TCS

If you have hybridomas that have been in continuous culture and are secreting antibody but you are uncertain if the culture is monoclonal, reclone the hybridomas in ClonaCell®-HY Liquid HAT Medium or in semi-solid ClonaCell®-HY Medium D (Catalog #03804) or ClonaCell®-TCS (Catalog #03814) to obtain a monoclonal culture.

A. Cloning by Limiting Dilution

Please refer to page 10, Method B: Cloning by limiting dilution, Steps 1b - 9b.

B. Cloning Using Semi-Solid Medium

Note: *ClonaCell®-HY Medium D contains HAT, while ClonaCell®-TCS does not contain selective agents. If the cells have been weaned off aminopterin, ClonaCell®-TCS is recommended.*

1. Culture the hybridomas in 10 mL of Medium E at a maximum cell density of 2 x 10^5 cells/mL. Prepare a cell suspension at a density of 200 cells/mL in Medium A or DMEM + 10% FBS.
2. Mix in a tube 9.5 mL of ClonaCell®-HY Medium D or ClonaCell®-TCS and 0.5 mL of hybridoma cell suspension (100 cells). Let sit at 37°C for 15 minutes.
3. Plate out the suspension in one petri dish as indicated in Section 2.8, Step 6a.

   10 - 14 days later, examine the plates for the presence of colonies visible to the naked eye. Assuming a viability of 50 - 80%, there will be 50 - 80 colonies in the dish.
4. Remove 15 - 20 colonies from the plate using a pipettor set to 10 µL and sterile 200 µL pipette tips. Pipette each clone into an individual well of a 96-well tissue culture plate containing 200 µL of Medium E. Incubate the plates at 37°C in 5% CO_2 for 1 - 4 days. Do not let cells overgrow.
5. Continue as indicated in Section 2.8, Steps 9a - 15a.

4.4 Appendix IV: Freezing and Thawing Cells

4.4.1 Freezing Hybridomas

1. Cells are cryopreserved at a concentration of 2 x 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 culture tube and cool on ice. Slowly add appropriate volume of DMSO and mix well. Filter sterilize solution using a 0.2 µm 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 x 10^6 cells/mL for cells cryopreserved at 2 x 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 freezer containers.

   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.
7. Place container in -70°C or -135°C freezer overnight.
8. Next day, remove frozen vials from the freezing container and store at -70°C, -135°C or in liquid nitrogen.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies

Version 1.0.0
February 2007
Catalog #29044

4.4.2 Thawing and Culturing of Cells (Parental Myeloma Cells, Hybridomas)

1. Store cells at -70°C or -135°C until ready to use.
2. Place 10 mL of Medium A into a sterile 15 mL tube.
3. Thaw cells quickly by agitating the vial in a 37°C waterbath.
4. Draw up the cell suspension in a 2 mL pipette and place in a 15 mL tube. Add dropwise 10 mL of culture medium. Centrifuge at 400 x *g* at RT or 37°C for 10 minutes.

   Wash step is required to remove DMSO.
5. Discard supernatant and resuspend the cells in 1 - 2 mL of culture medium.
6. Perform a manual nucleated cell count and calculate the volume of cells required.

   Usually 2 x 10^5 cells per T-75 cm^2 flask or 7 x 10^4 cells per T-25 cm^2 flask is sufficient.
7. Make up the volume with the appropriate culture medium:

   T-25 cm^2 flask; 10 mL

   T-75 cm^2 flask, 30 mL
8. Place the flasks in a humidified 37°C incubator containing 5% CO_2.
9. Grow cells to ~70 - 80% confluence (or to the cell density suggested by the protocol) and passage cells every 2 - 4 days.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies
Version 1.0.0
February 2007
Catalog #29044

5.0 References

1. Köhler G, Milstein C: Continuous cultures 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.

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: 1420-1424, 1979.

7. Pearson TW, Pinder M, Roelants GE, Kar SK, Lundin LB, Mayor-Withey KS, Hwett RS: Methods for derivation and detection of anti-parasite monoclonal antibodies. J Immunol Methods 34: 141-154, 1980.

8. Davis JM, Pennington JE, Kubler AM, 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.

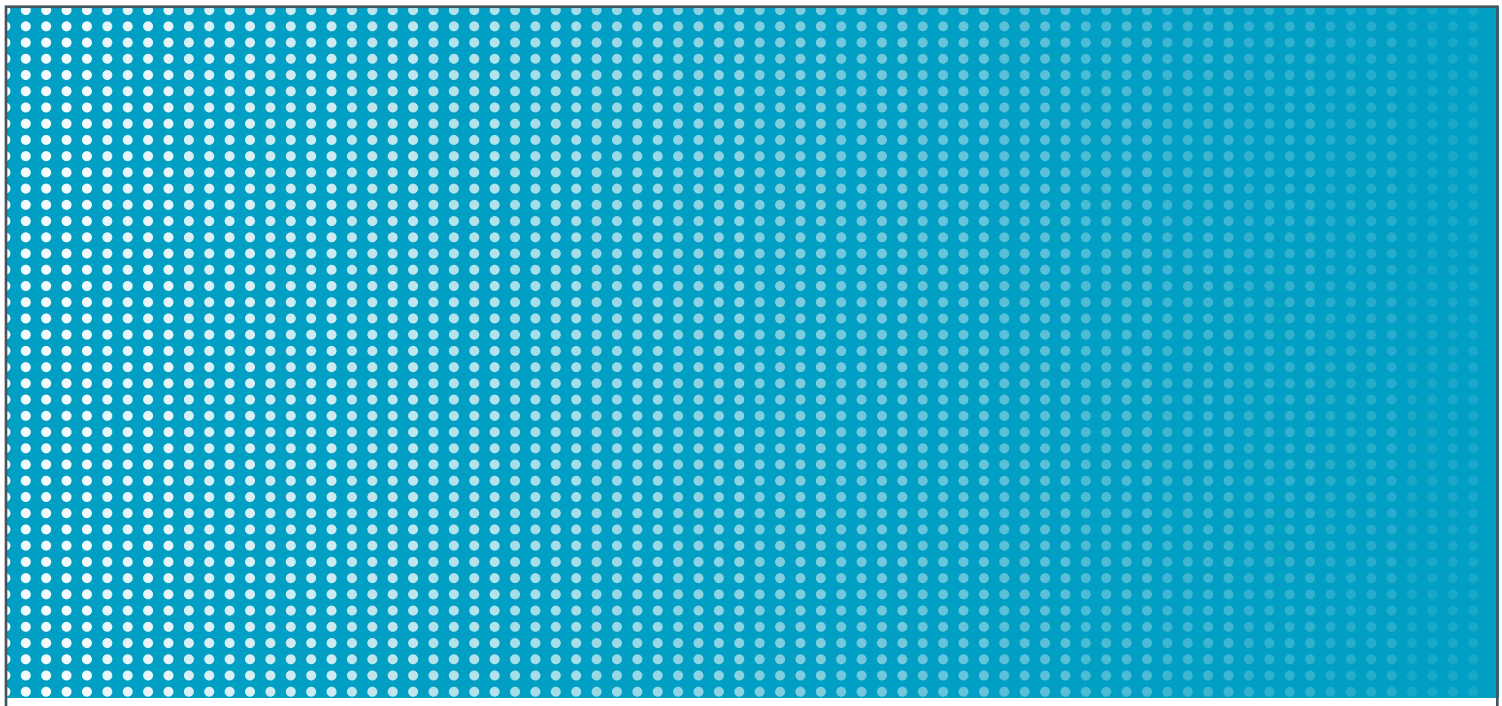
9. Coligan JE: Current Protocols in Immunology, Unit 2.5: Production of Monoclonal Antibodies. John Wiley & Sons, Inc, USA, 2006.

In North America
Tel: 1.604.877.0713
Fax: 1.604.877.0704
NA Toll Free Tel: 1.800.667.0322
NA Toll Free Fax: 1.800.567.2899
e-mail: info@stemcell.com
e-mail: orders@stemcell.com

In the United Kingdom
Tel: +44.(0).20.75.37.75.65
Fax: +44.(0).20.7915.54.08
Toll Free within United Kingdom:
Tel: 0800.731.27.14
Fax: 0800.731.27.13
E-mail: info@stemcellgb.com

In Europe
Tel: +33.(0).4.76.04.75.30
Fax: +33.(0).4.76.18.99.63
E-mail: info@stemcellfrance.com

StemCell Technologies
Version 1.0.0
February 2007
Catalog #29044



THE CELL EXPERTS™ | 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 • INFO@STEMCELL.COM

FOR FULL CONTACT DETAILS WORLDWIDE VISIT OUR WEBSITE

 MANUAL CATALOG #29044 VERSION 1.0.0