XP Media and CloneMedia for Mouse Hybridoma Generation

XP Media and CloneMedia for Mouse Hybridoma Generation provides a complete solution that supports all stages of hybridoma cell line development. The media are optimized to support the selection and growth of hybridoma clones using Molecular Devices ClonePix™ line of mammalian colony pickers, but they are also compatible with other appropriate methods.

Table 1-1: Available Kits

Item	Quantity	Part Number
XP Media and CloneMedia Complete Kit for Mouse Hybridoma Generation	1 each of the following part numbers: K8862, K8863, K8864, K8865, K8866, K8868	K8861

Table 1-2: Available Media

Item	Quantity	Part Number
XP Media Pre-Fusion Myeloma Growth Medium and Hybridoma Expansion Medium (without HT)	500 mL	K8862
XP Media Hybridoma Fusion Medium	500 mL	K8863
XP Media Hybridoma Fusion Recovery Medium	100 mL	K8864
CloneMedia Hybridoma Semi-Solid Selection and Cloning Medium (with HAT)	90 mL	K8865
XP Media Hybridoma Growth Medium (with HT)	500 mL	K8866
CloneMedia Hybridoma Semi-Solid Selection and Cloning Medium (without HAT)	90 mL	K8867
Hybridoma Polyethylene Glycol (PEG) for Cell Fusion	1.5 mL	K8868

Product manufactured by Molecular Devices, LLC.
1311 Orleans Drive, Sunnyvale, California, United States of America 94089.
Molecular Devices, LLC is ISO 9001 registered.

Contents

Chapter 1: About XP Media and CloneMedia for Mouse Hybridoma Generation

XP Media and CloneMedia for Mouse Hybridoma Generation is a complete solution that supports all stages of hybridoma cell line development. The media are optimized to support the selection and growth of hybridoma clones using a ClonePix system, but they are also compatible with other appropriate methods. The semi-solid CloneMedia method allows for selection and cloning of hybridoma cells shortly after fusion in one convenient step. This not only eliminates the possible masking of potentially valuable slow-growing clones by fast-growing clones, but also reduces or eliminates sub-cloning steps. HAT selection and cloning of hybridomas are accomplished simultaneously, which results in a substantial reduction in the cell line development timeline.

The advantages of CloneMedia compared to standard hybridoma cloning and selection methods are the following:

- Fewer manipulations are required, which significantly reduces the possibility of culture contamination.
- The use of Molecular Devices CloneDetect™ fluorescence detection agent enables screening for high antibody producers.
- The semi-solid CloneMedia method of cloning prevents the overgrowth of fast-growing clones, which allows for the selection of potentially valuable slow-growing clones.
- The semi-solid medium immobilizes the cells, thereby increasing the probability of single-cell colony formation and reducing additional sub-cloning steps.
- Hands-on time can be reduced when the workflow is combined with a ClonePix system. HAT selection and cloning of hybridomas are accomplished in one step, which minimizes both the time and the materials that are required.

XP Media and CloneMedia for Mouse Hybridoma Generation are optimized for use with CloneDetect, the ClonePix system, and Molecular Devices CloneSelect™ Imager system. The ClonePix system enables screening and isolation of high-secreting colonies, while the CloneSelect Imager system provides high-speed and label-free imaging that is tailored to monitor cell growth and verify monoclonality. By combining the ClonePix system and a three-dimensional semi-solid cell culture environment, you can rapidly and automatically screen a large and diverse population of clones for antibody production, pick the appropriate clones, and then deposit the clones directly into microplates that contain liquid medium to facilitate cell expansion and further characterization by downstream assays.

Mouse Hybridoma Generation Media

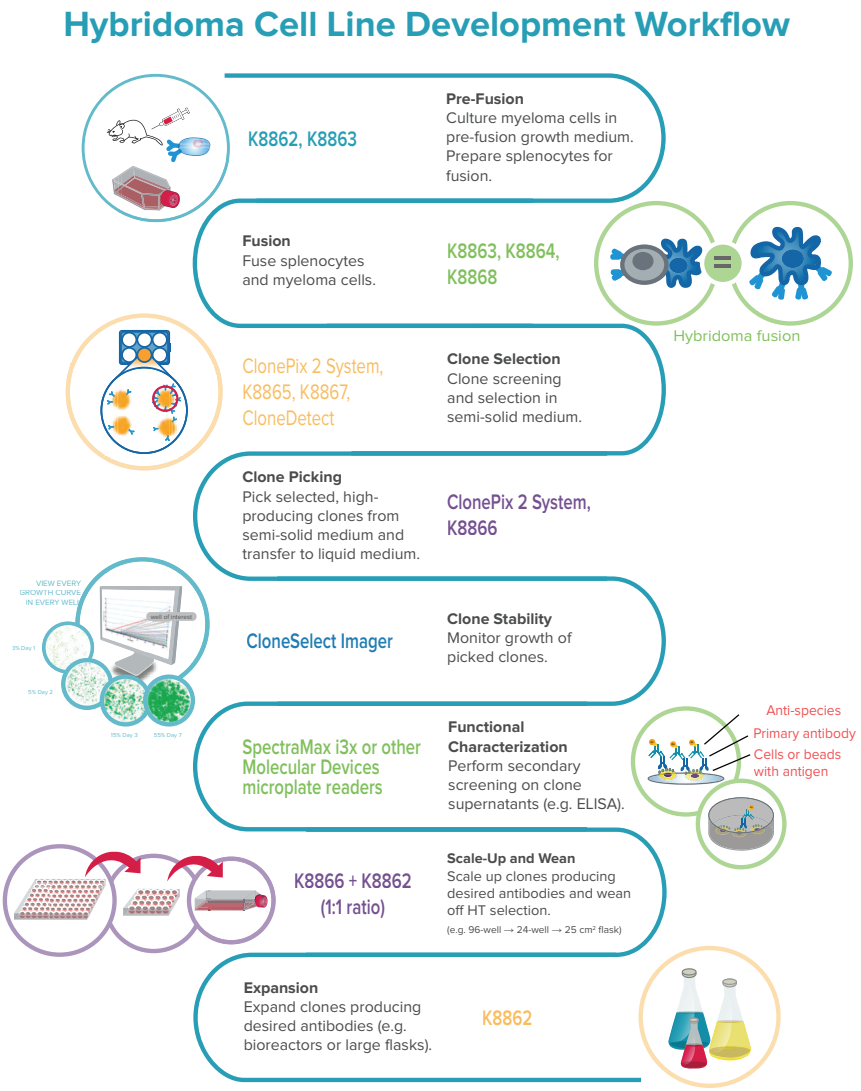


Figure 1-1: XP Media and CloneMedia for Mouse Hybridoma Generation: Hybridoma Cell Line Development Workflow

Recommended media and instruments to support all stages of hybridoma development and expansion workflows. The XP Media and CloneMedia Complete Kit together with the ClonePix and CloneSelect Imager systems improve the efficiency of selecting and cloning high-secreting hybridoma cells.

XP Media Pre-Fusion Myeloma Growth Medium and Hybridoma Expansion Medium (without HT) [#K8862]

Supports the growth of myeloma cells before fusion and it also supports the expansion of hybridoma clones. The medium does not contain hypoxanthine or thymidine (HT).

XP Media Hybridoma Fusion Medium [#K8863]

Used to wash cells before fusion and during the fusion process. This serum-free medium does not contain supplements to support growth and must not be used for cell growth or expansion of hybridoma clones.

XP Media Hybridoma Fusion Recovery Medium [#K8864]

Used to promote hybridoma viability after the fusion process, but before clone selection. This medium does not contain the selective reagents hypoxanthine, aminopterin, and thymidine (HAT).

CloneMedia Hybridoma Semi-Solid Selection and Cloning Medium (with HAT) [#K8865]

A semi-solid methylcellulose-based medium that contains the selective reagents hypoxanthine, aminopterin, and thymidine (HAT). The medium is used after fusion of splenocytes and myeloma cells to select and clone hybridomas in one step. The medium is optimized for colony formation. The single cell-derived hybridomas are immobilized in the semi-solid medium as they grow to form monoclonal colonies. Because the secreted product is visualized in the immediate vicinity of the producing colony, the hybridomas can easily be picked and screened, and then expanded for production of the selected antibody. The medium is equally suitable for fresh fusions and stable hybridoma cell lines.

XP Media Hybridoma Growth Medium (with HT) [#K8866]

A rich medium that has been optimized for hybridoma expansion following clone selection and colony picking. The medium contains hypoxanthine and thymidine (HT) and is used to wean hybridomas off aminopterin from the selection process. The medium supports better growth post-pick while maximizing antibody production.

CloneMedia Hybridoma Semi-Solid Selection and Cloning Medium (without HAT) [#K8867]

A semi-solid methylcellulose-based medium that does not contain any selection reagents. If appropriate selective reagent has been added, then the medium can be used after fusion of splenocytes and myeloma cells to select and clone hybridomas in one step. The medium is optimized for colony formation. The single cell-derived hybridomas are immobilized in the semi-solid medium as they grow to form monoclonal colonies. Because the secreted product is visualized in the immediate vicinity of the producing colony, the hybridomas can easily be picked and screened, and then expanded for production of the selected antibody.

Hybridoma Polyethylene Glycol (PEG) for Cell Fusion [#K8868]

Used for the fusion of mouse splenocytes and parental myeloma cells to generate hybridomas. Polyethylene Glycol is present as a 50% solution in Dulbecco's Modified Eagle's Medium (DMEM).

Note: Data that were collected using the different components in the hybridoma media portfolio are shown in Figure 1-2 and Figure 1-3 on the following two pages.

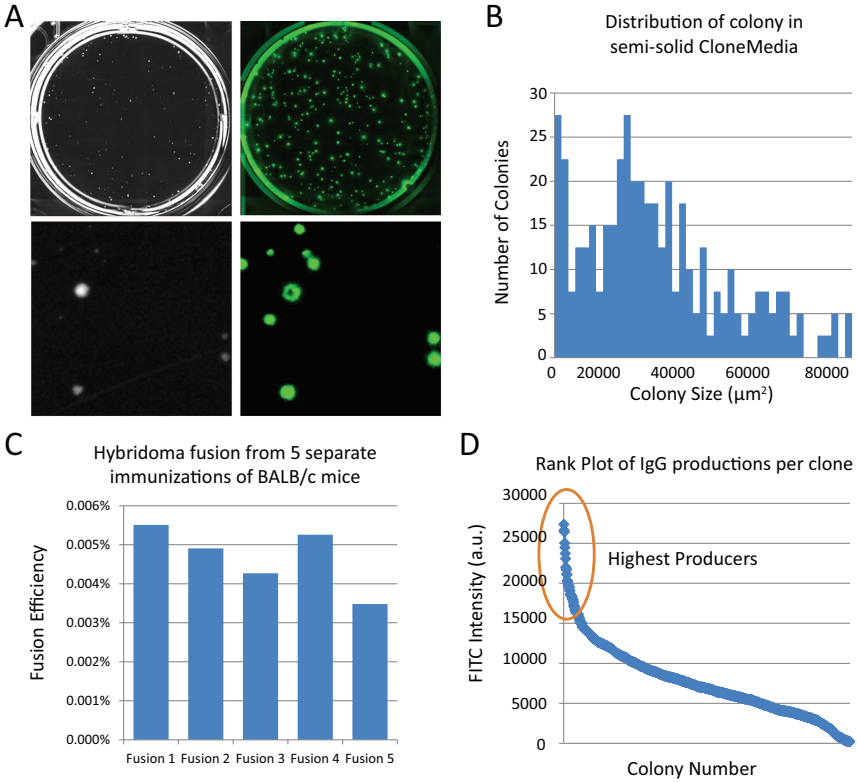
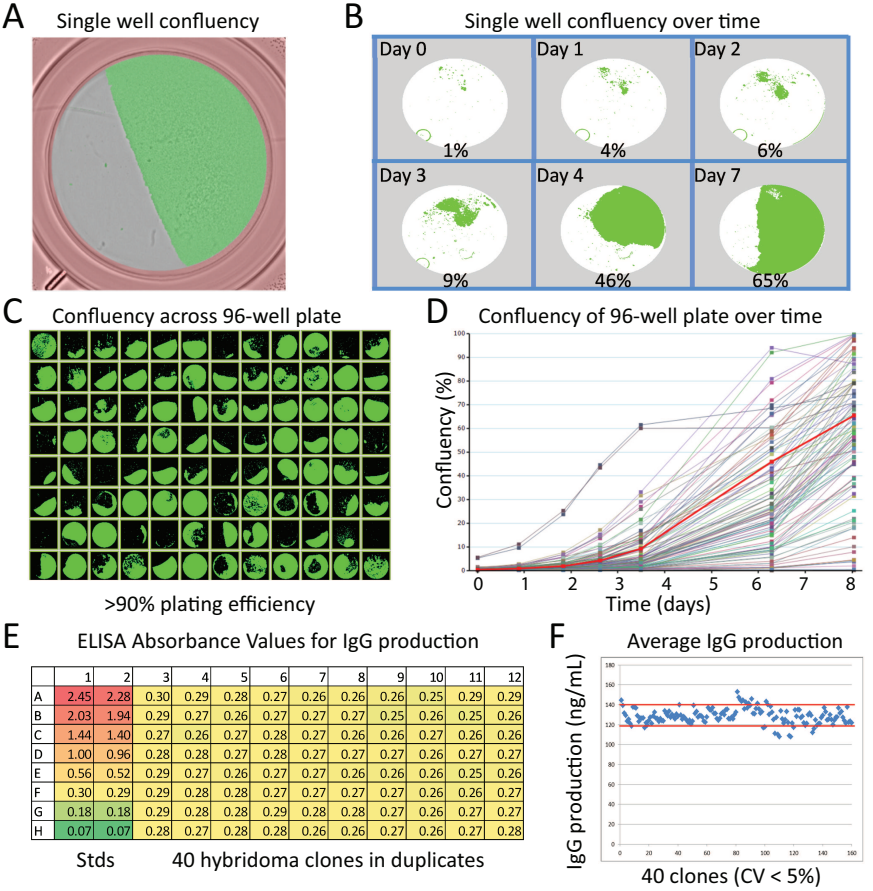


Figure 1-2: Hybridomas plated in CloneMedia Hybridoma Semi-Solid Selection and Cloning Medium (with HAT) in the presence of CloneDetect

A) Images of hybridomas were captured with the ClonePix system in white light (left panel) and FITC (right panel), after 7 days growth to determine growth and expression of IgGs, respectively.
B) Histogram plot of colony growth 7 days after plating.
C) Five individual hybridoma fusion experiments were conducted on BALB/c mice that were immunized to the same antigen to assess reproducibility of fusions in the XP Media and CloneMedia suite of products. Fusion efficiency was calculated by dividing the number of hybridoma colonies that were detected on the ClonePix system by the number of splenocytes that were used in fusion and plated in CloneMedia Hybridoma Semi-Solid Selection and Cloning Medium (with HAT).
D) Colonies that were grown in the presence of CloneDetect were ranked according to their FITC intensity, which indicated total IgG production, with the highest producers being picked for further characterization.



	1	2	3	4	5	6	7	8	9	10	11	12
A	2.45	2.28	0.30	0.29	0.28	0.27	0.26	0.26	0.26	0.25	0.29	0.29
B	2.03	1.94	0.29	0.27	0.26	0.27	0.27	0.27	0.25	0.26	0.25	0.26
C	1.44	1.40	0.27	0.26	0.27	0.28	0.27	0.26	0.26	0.27	0.26	0.26
D	1.00	0.96	0.28	0.28	0.27	0.28	0.27	0.27	0.27	0.26	0.27	0.27
E	0.56	0.52	0.29	0.27	0.26	0.27	0.27	0.26	0.26	0.26	0.25	0.26
F	0.30	0.29	0.29	0.28	0.28	0.27	0.27	0.27	0.27	0.26	0.26	0.27
G	0.18	0.18	0.29	0.28	0.28	0.29	0.28	0.28	0.27	0.26	0.27	0.27
H	0.07	0.07	0.28	0.27	0.28	0.28	0.26	0.26	0.27	0.26	0.27	0.28

Figure 1-3: Sub-cloning of stable HFN7.1 hybridomas in XP Media Hybridoma Growth Medium (with HT)

A) Example image of a single well 7 days post-picking, collected on the CloneSelect Imager. Green overlay indicates confluency detection by the software.
B) Cell confluency of a single well measured over time.
C) Software detection of cell confluency across a 96-well plate allowing for a quick visualization of plating efficiency. The initial confluency of all wells was <1%. 87 out of 96 wells grew to a confluency >5% after 7 days for a >90% plating efficiency. Because slow growing clones might be classified as non-growing using the > 5% confluency criteria, the actual plating efficiency might be even greater.
D) Growth curves of all the hybridoma clones from a single plate. 40 clones were selected based on their confluency for assessment of IgG production by ELISA.
E) Absorbance values collected from an ELISA plate assessing total IgG production.
F) IgG production plotted per well, shown in blue, with red lines indicating 2 s.d. away from the mean. Because these are stable hybridomas, we do not expect a large variation in the total amount of IgG produced per cell, which is confirmed by <5% CV across all the clones that were tested.

Chapter 2: Materials and Equipment

Available Kits and Media

Table 2-1: Available Kits

Item	Quantity	Part Number
XP Media and CloneMedia Complete Kit for Mouse Hybridoma Generation	1 each of the following part numbers: K8862, K8863, K8864, K8865, K8866, K8868	K8861

Table 2-2: Available Media

Item	Abbreviated Name	Quantity	Part Number
XP Media Pre-Fusion Myeloma Growth Medium and Hybridoma Expansion Medium (without HT)	Expansion Medium (without HT)	500 mL	K8862
XP Media Hybridoma Fusion Medium	Fusion Medium	500 mL	K8863
XP Media Hybridoma Fusion Recovery Medium	Recovery Medium	100 mL	K8864
CloneMedia Hybridoma Semi-Solid Selection and Cloning Medium (with HAT)	Semi-Solid Medium (with HAT)	90 mL	K8865
XP Media Hybridoma Growth Medium (with HT)	Growth Medium (with HT)	500 mL	K8866
CloneMedia Hybridoma Semi-Solid Selection and Cloning Medium (without HAT)	Semi-Solid Medium (without HAT)	90 mL	K8867
Hybridoma Polyethylene Glycol (PEG) for Cell Fusion	PEG	1.5 mL	K8868

Note: Reagents can be ordered separately. If you are using alternate hybridoma selection methods, then Semi-Solid Selection Medium (without HAT) (Part #K8867) is available. You must add agents for hybridoma selection to this medium before use.

Materials Required but Not Provided

Consumables

- General laboratory supplies and tools (for example, serological pipettes, pipet aid, and pipettors)
- 6-well suspension culture plates (non-TC treated) or OmniTrays
- 96-well suspension culture plates (non-TC treated) (Molecular Devices) P/N 655185
- Sterile tissue cultures flasks with vented caps (T-25 cm^2 and T-75 cm^2)
- 24-well sterile tissue culture plates
- 12 mL syringe
- Blunt end 16 gauge needle

Fusion

- Forceps
- Fine scissors
- 3 mL syringe
- Fine mesh screen or disposable cell strainer
- Myeloma cell line (for example, Sp2/0 or X63Ag8.653)
- Primed mouse 1 to 4 days after final antigen boost

Reagents

- Materials for doing functional characterization assay (for example, ELISA, Western Blot, and so on)
- Trypan blue or other viability dye
- Sterile, distilled water
- Dimethylsulfoxide (DMSO)
- Fetal bovine serum (FBS)
- 95% ethanol

Instrumentation

- Inverted microscope or imaging system (for example, ClonePix or CloneSelect Imager systems)
- Hemocytometer or other cell counter

Storage and Handling

WARNING! Media can contain chemicals that are harmful. Exercise care when handling media as described in the related safety data sheet (SDS or MSDS). The safety data sheet is available in the Knowledge Base on the Molecular Devices support web site: www.moleculardevices.com/support

Storage

- Store all XP Media and CloneMedia for Mouse Hybridoma Generation at –20°C. When stored as instructed, the product is stable until the expiration date that is listed on the Certificate of Analysis.
- Store PEG at 2°C to 8°C. When stored as instructed, the product is stable until the expiration date that is listed on the Certificate of Analysis.

Thawing

- Before use, thaw all XP Media and CloneMedia for Mouse Hybridoma Generation at room temperature overnight. Do not shake contents until completely thawed. Allow all media to adjust to room temperature before using.

Note: During the assay, some XP Media for Mouse Hybridoma Generation must be warmed to 37°C before use. This is noted where appropriate.

CAUTION! Do *not* thaw the Semi-Solid Medium (with or without HAT) in a water bath or pre-warm it to 37°C.

- Before use, pre-warm the PEG to 37°C.

Storage after Thawing

When thawed, XP Media and CloneMedia for Mouse Hybridoma Generation can be stored at 2°C to 8°C for a limited time.

- Expansion Medium (without HT) (Part #K8862) can be stored at 2°C to 8°C for up to 1 month.
- Fusion Medium (Part #K8863) can be stored at 2°C to 8°C for up to 4 months.
- Recovery Medium (Part #K8864), Semi-Solid Medium (with or without HAT) (Part #K8865 and #K8867), and Growth Medium (with HT) (Part #K8866) can be stored at 2°C to 8°C for up to 14 days.

Compatible Molecular Devices Instruments and Reagents

- ClonePix system: The ClonePix system enables screening and picking of high-secreting colonies. The ability of the ClonePix software to screen and rank secreting colonies before picking significantly reduces the cell line development timeline and maximizes monoclonality verification from the beginning of the process.
- CloneSelect Imager system: The CloneSelect Imager provides high-speed label-free imaging to monitor cell growth and verify monoclonality.
- CloneDetect: The CloneDetect fluorescence detection agent is used to screen clones for antibody secretion.

Chapter 3: Mouse Hybridoma Generation Protocol

Unless noted otherwise, do all procedures using sterile techniques in a certified biosafety cabinet. Do the procedures in the following order:

Immunization of BALB/c Mice

To allow BALB/c mice to develop a robust immune response before generating hybridomas, immunize them with antigen 6 to 10 weeks before fusion. To simplify the screening of hybridomas, Molecular Devices recommends that you immunize the mice with a pure antigen. You can, however, use complex antigenic mixtures. Generally, you do a fusion 1 to 4 days after the final immunization boost.

Note: For an example of a typical injection schedule for immunizing BALB/c mice, see BALB/c Mouse Immunization on page 20.

Myeloma Cell Preparation

The parental myeloma cells that you use to make the hybridomas must meet the following criteria:

- The cells must match the strain of mouse that you have immunized. For example, if you have immunized BALB/c mice, then the myeloma cells must be of BALB/c origin.
- The cells must not secrete any of their own immunoglobulin chains.
- The cells must be mycoplasma free.
- The cells must fuse well and allow the formation of stable hybridomas that continually secrete specific monoclonal antibodies.

Note: 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. Store the cells in liquid nitrogen until you are ready for production.

Myeloma cell preparation consists of thawing and culturing the cells, and then harvesting the cells.

Thawing and Culturing the Parental Myeloma Cells

1. Pre-warm the Expansion Medium (without HT) to 37°C.

   Note: See Table 2-2: Available Media on page 9.

2. Agitate the frozen vial in a 37°C water bath to thaw the myeloma cells quickly.

3. Do the following in the order indicated to wash the thawed cells and remove any DMSO:
   - Use a 2 mL pipette to draw up the cell suspension, and then add the suspension to a 15 mL tube.
   - Add 10 mL of the pre-warmed Expansion Medium (without HT) drop-wise to the tube.
   - Centrifuge at 400 x g for 10 minutes.
4. Discard the supernatant, and then re-suspend the cells in 1 mL to 2 mL of the pre-warmed Expansion Medium (without HT).
5. Use a hemocytometer or other cell counter with a viability dye to determine the viable cell concentration, and then calculate the volume of cells that is required to seed at a cell density of ~5 x 10^4 viable cells/mL.
6. Use the appropriate volume of pre-warmed culture medium to give a final volume of 10 mL/T-25 cm^2 flask or 30 mL/T-75 cm^2 flask.
7. Place the flask in a 37°C incubator with 5% CO_2 in air with 95% humidity.
8. Culture the cells in the culture medium for at least one week before fusion to ensure that the cells are well adapted to the medium. Seed cells at a density of approximately 5 x 10^4 cells/mL and passage every 2 days.
   During the culturing of the cells, and before fusion, note the following:
   - If cells grow beyond 8 x 10^5 cells/mL, then passage them at least twice to return them to early-mid log phase growth.
   - Test the cells for mycoplasma. If the myeloma cells are positive for the presence of mycoplasma, then do not proceed with fusion. You must instead thaw a new vial or obtain new myeloma cells.
   - Calculate the cell growth rate at every passage. The day before the fusion, count the viable cells and split so that at least 2 x 10^7 parental myeloma cells are available for fusion.

Note: The recommended cell density for fusion is 2 x 10^5 cells/mL. Only 100 mL of these cells are required, but culture 200 mL to ensure sufficient cell numbers for fusion.

Harvesting the Parental Myeloma Cells

To ensure that the parental myeloma cells are not sitting for an extended period of time, you can harvest the cells at the same time that you are preparing the spleen cells or after. To ensure that all the serum that is adhering to the cells is removed, you must wash the cells with Fusion Medium. Otherwise, the PEG cannot fuse the cell membranes and the fusion frequency drops drastically.

To harvest the parental myeloma cells, do the following:

1. Centrifuge the cells in a 50 mL conical centrifuge tube at room temperature (RT) or 37°C at 300 x g for 10 minutes.
2. Do the following 3 times to wash the cells: Add 30 mL of Fusion Medium, and then repeat the centrifugation.
3. Use a pipette to remove the supernatant, and then re-suspend the cell pellet in 25 mL of Fusion Medium.
4. Use a viability stain such as Trypan Blue to count live cells.

Note: The viability of parental myeloma cells should be > 95%.

5. Calculate the volume of cell suspension that contains 2 x 10^7 viable cells.
6. Keep cells at RT or 37°C until fusion.

Splenocyte Preparation

To prepare the splenocytes for fusion, you must disaggregate the spleen into a single cell suspension, wash the cells, and then dilute the cells to the appropriate number.

Disaggregating the Spleen into a Single Cell Suspension

1. Sacrifice an immunized mouse according to the procedures that your institution recommends, and then wash the fur with 95% ethanol.
2. Clip the fur, and then pull back to expose the chest.
3. Remove the spleen and place it in a sterile Petri dish that contains 5 mL of Expansion Medium (without HT).
4. Trim off any large pieces of fatty tissue.
5. Disaggregate the spleen into a single cell suspension. A suggested method of disaggregation is the following:
   - Transfer the spleen to a screen that has been placed on top of a 50 mL conical centrifuge tube.
   - Use the plunger of a sterile 3 mL syringe to grind the cells out of the spleen.
   - Rinse the screen with Fusion Medium 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 *do not* cause the solution to foam.

Washing the Splenocyte Suspension

To ensure that all the serum that is adhering to the cells is removed, you must wash the splenocyte suspension with Fusion Medium. Otherwise, the PEG cannot fuse the cell membranes and the fusion frequency drops drastically.

1. To wash the splenocyte suspension, do the following three times in the order indicated:
   - Add 30 mL of Fusion Medium to the suspension.
   - Centrifuge at 400 x g at room temperature (RT) or 37°C for 10 minutes.
   - Use a pipette to remove the supernatant being careful not to disturb the cell pellet.
2. After the final wash, re-suspend the cells in 25 mL Fusion Medium.

Counting and Diluting the Splenocyte Cells

1. Prepare a 1:1 dilution of the cells in Trypan Blue. For example, dilute 10 µL of the cell suspension with 10 µL of Trypan Blue.
2. Use a hemocytometer or other cell counter to count the total number of *viable* cells in the diluted sample.

Note: If the toxicity of Trypan Blue is a concern, then a 1:10 dilution of the cells in 3% acetic acid with methylene blue can be used in its place.

3. Calculate the volume of cell suspension that contains 1 x 10^8 cells.
4. Place cells at RT or 37°C until fusion.

Fusion

Two methods of fusion are available: **Gentle** and **Quick**. With either method, on the day of the fusion, you *must* place Semi-Solid Medium (with HAT) at room temperature and thaw overnight.

Gentle Fusion Method

Before doing a **Gentle** fusion, prepare a 37°C water bath.

1. Pre-warm all properly thawed PEG, Expansion Medium (without HT), Fusion Medium, and Recovery Medium to 37°C. (See Thawing on page 11.)
2. Combine 2 x 10^7 parental myeloma cells and 1 x 10^8 viable splenocytes in a 50 mL conical centrifuge tube, and then centrifuge for 10 minutes at 400 x g.
3. Aspirate off the supernatant.

CAUTION! To avoid dilution of PEG, complete removal of the supernatant is essential.

4. Gently tap the bottom of the tube that contains the cell pellet that was produced in Step 2 to disrupt the pellet.

CAUTION! The pellet must be completely disrupted for optimal fusion.

5. Use a 1 mL pipette to slowly add 1 mL of PEG drop-wise to the pellet over a period of 1 minute *without* stirring.
6. Use the pipette tip to continually stir the cells gently for 1 minute.
7. Add 4 mL of Fusion Medium to the fusion mixture, continually stirring as before, for 4 minutes.
8. Slowly add 10 mL of Fusion Medium to the fusion mixture.
9. Incubate the fusion mixture for 15 minutes in the water bath at 37°C.
10. Slowly add 30 mL of Expansion Medium (without HT) to the fusion mixture, and then centrifuge the mixture at 400 x g for 7 minutes.
11. Discard the supernatant, and then wash the cell pellet with 40 mL of Expansion Medium (without HT) to ensure that all the PEG is removed.
12. Slowly re-suspend the cell pellet in 10 mL of Recovery Medium.
13. Transfer the cell suspension to a T-75 tissue culture flask that contains 20 mL of Recovery Medium for a total culture volume of 30 mL.
14. Incubate the culture for 16 to 24 hours at 37°C in a 5% CO_2 atmosphere.

Quick Fusion Method

1. Pre-warm all properly thawed PEG, Expansion Medium (without HT), Hybridoma Fusion Medium, and Recovery Medium to 37°C. (See Thawing on page 11.)
2. Combine 2 x 10^7 parental myeloma cells and 1 x 10^8 viable splenocytes in a 15 mL conical centrifuge tube, and then centrifuge for 10 minutes at 400 x g.
3. Aspirate off the supernatant.

CAUTION! To avoid dilution of PEG, complete removal of the supernatant is essential.

4. Gently tap the bottom of the tube that contains the cell pellet that was produced in Step 2 to disrupt the pellet.

CAUTION! The pellet must be completely disrupted for optimal fusion.

5. Use a 1 mL pipette to add 0.5 mL of PEG drop-wise to the pellet.
6. Centrifuge the mixture at 133 x g at room temperature or 37°C for 3 minutes.
7. Aspirate off all PEG.

CAUTION! During Step 7, not all cells will form a pellet, as some clump in the PEG. Do not aspirate the clumped cells. Work quickly because the cells must not be exposed for too long to PEG, or the cell viability drops.

8. While gently swirling the tube to re-suspend the cells, carefully add 5 mL of Fusion Medium drop-wise to the pellet.
9. While continuing to gently swirl the tube, slowly add 5 mL of Recovery Medium to the re-suspended cell solution.
10. Transfer the cell suspension to a T-75 tissue culture flask that contains 20 mL of Recovery Medium for a total culture volume of 30 mL.
11. Incubate the culture for 16 to 24 hours at 37°C in a 5% CO_2 atmosphere.

CAUTION! Some clumps of cells might still be present at this point, and they dissolve overnight. Be gentle with these cells.

Plating and Cloning of Hybridomas

Plating and cloning of hybridomas consists of preparing the required Semi-Solid Medium (with HAT), and then plating the cells in the medium to produce the clones. Start plating and cloning of hybridomas the day after the fusion.

Note: The following procedure focuses on Semi-Solid Medium (with HAT) [#K8865]. If you are using alternate selection agents with Semi-Solid Medium (without HAT), then adjust the procedure accordingly.

Preparing Cells and Semi-Solid Medium (with HAT)

1. On the day after the fusion, shake the properly thawed Semi-Solid Medium (with HAT) vigorously to mix the contents well, and then let warm to room temperature (RT).
2. Transfer the fused cell suspension into a 50 mL conical tube, and then centrifuge for 10 minutes at 400 x g at RT or 37°C.
3. Aspirate the supernatant.
4. Re-suspend the cells in 10 mL of Recovery Medium, and then set aside.

5. To the bottle that contains 90 mL of Semi-Solid Medium (with HAT), add any other required components, for example, CloneDetect fluorescence detection agent or antibiotics. Note the following for this step:
   - The total volume of other required components must not exceed 10 mL.
   - The final volume of the solution must be 100 mL. If required, you can add Recovery Medium to bring the final volume to 100 mL.
   - You can add CloneDetect reagent or other fluorescently labeled antibodies only at this step.

CAUTION! After a fluorescence detection agent is added to the Semi-Solid Medium (with HAT), you must protect the bottle from direct light.

6. Shake the bottle vigorously for approximately 30 seconds to mix the contents thoroughly.
7. Allow the medium to rest for approximately 10 minutes at RT for the bubbles to dissipate.

Note: Any remaining small bubbles disperse in the plate during the culture period.

Cell Plating in Semi-Solid Medium (with HAT)

The correct density of colonies in the culture dish is key to the success of colony selection and the automated picking process. It is therefore crucial to optimize the seeding densities thoroughly. The seeding densities that are required to achieve an optimal density of colonies depend on the inherent plating efficiency of the cells that are used and on the viability and growth phase of the cell-suspension culture at the time of plating. The following ranges of seeding densities can serve as a guideline for your optimization:

- Stable and robust hybridomas: 100 to 200 cells/mL
- Fresh fusions: 1 x 10^3 to 1 x 10^6 cells/mL

Note: When plating fresh fusions, the optimal seeding density is highly dependent on the fusion efficiency and the kinetics and efficiency of selection. The successful selection of clones might require the optimization of selection agents. Cells can also require a period of recovery before seeding in a semi-solid medium. To ensure clonality, you might have to gently pipette the cell suspension up and down to dissociate the cells. Care must be taken not to damage the cells.

CAUTION! It is crucial that appropriate culture plastics are used. Plate cells into non-TC-treated culture dishes.

1. Add the appropriate number of cells to the Semi-Solid Medium (with HAT). Use the cells that were set aside in Step 4 of Preparing Cells and Semi-Solid Medium (with HAT) on page 16.
2. Invert the bottle gently at least 10 times to thoroughly mix the cells and medium.
3. Using a 12 mL syringe and 16 gauge blunt-end needle, aseptically dispense 2 mL of the mixture into each well of a 6-well plate or 18 mL per tray into an OmniTray.

CAUTION! Because methylcellulose is a viscous solution and it adheres to the pipette walls, you cannot use a pipette to accurately dispense it.

4. Tilt each plate to evenly distribute the medium to cover the bottom of the plate.

CAUTION! Avoid the introduction of bubbles during plating.

5. If you are *not* using a 6-well plate, then go to the next step. Otherwise, to ensure that the semi-solid medium remains well hydrated, place 4 mL of sterile water or sterile buffer in the area between the wells.
6. Incubate the plates at 37°C in a 5% CO_2 atmosphere until significant confluency is reached.

Note: Molecular Devices recommends monitoring growth with an automated imager such as the CloneSelect Imager, or with a bright field microscope.

CAUTION! To prevent the formation of runny or hazy colonies, do not disturb the plates for at least the first 10 days up to 14 days. Culture conditions are very important to ensure optimal growth of hybridoma colonies. Molecular Devices recommends using a water-jacketed incubator. Open and close the incubator door carefully to avoid shaking.

Tip: If appropriate, you can prepare an extra plate as an "observation plate" to monitor colony growth during the incubation period. Allow the observation plate to incubate undisturbed for at least 4 days before viewing or imaging to allow the single cells to start to divide and grow. Beyond 4 days, you can observe this plate daily until a suitable colony size is reached.

Harvesting Colonies

1. 10 to 14 days after the cells have been plated in Semi-Solid Medium (with HAT), you can use the ClonePix or CloneSelect Imager systems, or a bright field microscope, to image the colonies. A typical fusion produces 1000 or more colonies over ten 6-well plates or a few OmniTrays.

Note: The CloneMedia have been optimized for use with the ClonePix or CloneSelect Imager systems. These systems provide label-free imaging to confirm the progress of colony growth and determine an appropriate window for picking clones.

2. For each isolated colony that is to be picked, use a ClonePix system, or a manual pipette that is set to 10 µL, to pick the colony, and then transfer the colony to a 96-well tissue culture plate that contains 200 µL/well Growth Medium (with HT).

3. Do one of the following to re-suspend the colony:
   - If you are using a ClonePix system, then add a dispersal step to the instrument protocol.
   - If you are using a manual pipette, then set the pipette to 150 µL and pipette the contents of the well up and down several times.

Note: You do not need to re-suspend the colony into a perfect single-cell suspension but disperse the colony sufficiently to get good growth.

Tip: After you have transferred all the selected colonies to 96-wells, you can use a multi-channel pipettor to re-suspend the colonies. You must use a new sterile tip for each clone to maintain the clonality of the colony.

4. Incubate the plates at 37°C in 5% CO_2 for 1 to 7 days without feeding.
5. Transfer 150 µL of supernatant from each hybridoma to a separate well on a new 96-well plate, and then analyze by using an assay system and a Molecular Devices multi-mode plate reader that is appropriate for the antigen involved, for example, ELISA, Western Blotting, and so on.
6. Add 150 µL of fresh Growth Medium (with HT) to every well of the original hybridoma-containing plates.
7. Gently re-suspend those hybridomas that showed a positive response in Step 5. If you are using a fluorescent detection agent, then gently re-suspend the hybridomas that showed a positive response in Step 2 also.
8. Transfer 100 µL of cells to each of 2 wells of a 24-well plate, with each well containing 1 mL of Growth Medium (with HT).
9. After the cells have grown to a suitable density (approximately 4 x 10^5 cells/mL), freeze the cells from one well. See Freezing Harvested Hybridomas on page 21.
10. To adapt the cells to growth without HT, expand the remaining positive clones in a T-25 tissue culture flask that contains a 1:1 mixture of 5 mL of Expansion Medium (without HT) and 5 mL of Growth Medium (with HT).

Tip: Molecular Devices recommends that you keep a sample of cells in 100% Growth Medium (with HT) as a backup in case the cells do not adapt well to the 1:1 mixture.

11. After the cells have grown to a suitable density (approximately 4 x 10^5 cells/mL), use a pipette to transfer 3 mL to 10 mL of cell culture into 20 mL of Expansion Medium (without HT) in a T-75 cell culture flask.
12. Adjust the volume of cells to ensure that the final cell concentration is between 1 x 10^4 to 5 x 10^4 cells/mL.

Going forward, maintain expanded hybridomas in 100% Expansion Medium (without HT) at a concentration of 5 x 10^4 to 5 x 10^5 cells/mL. To secure the supply of hybridoma, you can freeze more aliquots of cells at this point. See Freezing Harvested Hybridomas on page 21.

Tip: Molecular Devices recommends recloning if you suspect that a culture is not monoclonal, which generally occurs if the cell density in the plates was high and there is a possibility that cells of two or more colonies were therefore harvested. Molecular Devices also recommends recloning for hybridomas that have been in continuous culture for an extended time, in particular if antibody production has declined and selection of high antibody secreting subcultures is required. See Recloning Hybridomas on page 22.

Appendix A: BALB/c Mouse Immunization

The following provides an example of a typical injection schedule for immunizing BALB/c mice before the fusion. This is a suggested injection schedule only and the actual timing can vary depending on the antigen used for the immunization and other factors. Generally, you do the fusion one to four days after the final immunization boost.

Tip: Pre-warm the mouse with a heat lamp and apply a topical anesthetic to its tail before sample collection or antigen injection.

1. To collect a sample of serum or plasma before immunization to use as a baseline control for antibody screening, do the following in the order indicated:
   - Bleed the mice by cutting approximately 1 mm to 2 mm off the tip of the tail.
   - Collect 100 µL to 200 µL of blood in a heparin-coated capillary tube, and then prepare plasma.
   - Add 0.1% sodium azide to the serum and store at –20°C.
   - Thaw just before use.
2. Inject 2 to 4 adult BALB/c mice intraperitoneally with either of the following:
   - 20 µg to 100 µg of purified antigen in a total volume of 200 µL of a 1:1 emulsion of antigen in saline or adjuvant.
   - 100 µg to 200 µg of antigen mixture in a total volume of 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. 10 to 30 days after the first injection, repeat it for the second injection.
4. 10 to 14 days after the second injection, cut 1 mm to 2 mm from the tip of the tail and collect 100 µL to 200 µL of blood in a heparin-coated capillary tube.
5. Prepare plasma from the blood sample, and then measure the antibody levels using the appropriate technique such as ELISA, immunofluorescence, flow cytometry, immunoblotting, and so on.

   Note: Be sure to compare to the pre-immunization serum from the same mouse.
6. Select the mouse that has the highest antibody titres for further boosting with antigen. Continue to give injections at two week intervals until a good titre of antibody is obtained.
7. 1 to 4 days before the day of the fusion, depending on factors such as route of immunization, boost the selected BALB/c mouse intravenously through the tail vein.

   Note: Generally, antigen is dissolved or suspended in saline and a maximum of 200 µL are injected.

Appendix B: Freezing Harvested Hybridomas

When you freeze harvested hybridomas, cryopreserve the cells at a concentration of 2 x 10^6 cells per vial. To freeze aliquots of harvested hybridomas, do the following:

1. Label the required number of sterile 2 mL cryovials (1.8 mL capacity).
2. To prepare a 20% DMSO solution in FBS, do the following in the order indicated:
   - Place FBS in a 50 mL conical tube, and then cool on ice.
   - Slowly add the appropriate volume of DMSO, and then mix well.
   - Use a 0.2 µm filter to filter-sterilize the solution and keep on ice.
3. Re-suspend the harvested cells in cold FBS at 4 x 10^6 cells/mL (2x the required final cell concentration).
4. Slowly add the 20% DMSO in FBS solution at a ratio of 1:1 to the tube that contains the harvested cells. Continue to mix during the addition.
5. Transfer 1 mL of cells in freezing medium to each cryovial. The final cell suspension is 2 x 10^6 cells/mL in 90% FBS to 10% DMSO.
6. Place cryovials immediately into freezing containers.

CAUTION! 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 the instructions from the manufacturer.

Appendix C: Recloning Hybridomas

Molecular Devices recommends recloning if you suspect that a culture is not monoclonal. This generally occurs if the cell density in the plates was high and there is a possibility that cells of two or more colonies were therefore harvested. Molecular Devices also recommends recloning for hybridomas that have been in continuous culture for an extended time, in particular if antibody production has declined and selection of high antibody secreting subcultures is required.

To reclone hybridomas, do the following:

1. Culture the hybridomas in 10 mL of Growth Medium (with HT) at a maximum cell density of 2 x 10^5 cells/mL.
2. Prepare a cell suspension at a density of 100 cells/mL in Expansion Medium (without HT).
3. In a 50 mL conical tube, mix 18 mL of Semi-Solid Medium (with HAT) that contains the appropriate amount of CloneDetect or another fluorescent detection agent and 2 mL of hybridoma cell suspension (200 cells).
4. Plate out the suspension in one OmniTray as described in Plating and Cloning of Hybridomas on page 16.
5. 10 to 14 days after the cells have been plated in Semi-Solid Medium (with HAT), use the ClonePix or CloneSelect Imager systems, or a bright field microscope, to image the colonies.

Note: Assuming a plating efficiency of 50% to 80%, 100 to 160 colonies will be in the tray.

6. For each colony (clone) that shows good antibody production, do the following: Use the ClonePix system to transfer the clone into an individual well of a 96-well tissue culture plate that contains 200 µL of Growth Medium (with HT).
7. Incubate the plates at 37°C in 5% CO_2 for 1 to 4 days.

CAUTION! Do not let the cells overgrow.

8. Continue the procedure starting at Step 5 in Harvesting Colonies on page 18.

Obtaining Support

Molecular Devices is a leading worldwide manufacturer and distributor of analytical instrumentation, software, and reagents. We are committed to the quality of our products and to fully supporting our customers with the highest possible level of technical service.

Our support web site, www.moleculardevices.com/support, has a link to the Knowledge Base with technical notes, software upgrades, safety data sheets, and other resources. If you do not find the answers you are seeking, follow the links to the Technical Support Service Request Form to send an email message to a pool of technical support representatives.

You can contact your local representative or contact Molecular Devices Technical Support by telephone at 800-635-5577 (U.S. only) or +1 408-747-1700. In Europe call +44 (0) 118 944 8000.

Please have the product name, part number, and lot number available when you call.

Contact Us

Phone: +1-800-635-5577
Web: moleculardevices.com
Email: info@moldev.com

Visit our website for a current listing of worldwide distributors.

R3647 A