ClonaCell™-CHO

Semi-Solid Cloning Testing Guidelines

STEMCELL™ TECHNOLOGIES

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1.0 Introduction

1.1 Using This Document

Thank you for testing ClonaCell™ semi-solid media for Chinese hamster ovary (CHO) cells. Because each CHO cell line and cell line development project is unique, it is important to optimize the semi-solid cloning protocol for your specific laboratory and/or application in order to obtain the best results. This document provides step-by-step guidelines for testing ClonaCell™ media. The recommendations provided here are based on the range of cell lines and laboratory conditions we have commonly encountered with our customers.

This document provides guidelines for testing the products listed in Table 1. For more information and standard cloning protocols for each of these products, please refer to the appropriate Product Information Sheet (PIS), available at **www.stemcell.com**.

This document contains:

- Guidelines for choosing the right ClonaCell™ medium
- Recommended pre-cloning cell culture conditions
- Information on incubator conditions, cultureware and other equipment
- Detailed testing protocols
- Additional suggestions for protocol optimization

Table 1. ClonaCell™ semi-solid media products

PRODUCT NAME	DESCRIPTION	CATALOG #
ClonaCell™-TCS Medium	Serum-containing medium for selection and cloning of CHO cells and other cell lines	03814
ClonaCell™-CHO CD Medium	Chemically defined, protein-free medium for selection and cloning of CHO cells	03815
ClonaCell™-CHO ACF Medium	Animal component-free medium for selection and cloning of CHO cells	03816
ClonaCell™ FLEX[1]	Semi-solid base medium for cloning a variety of cell types	03818

1 ClonaCell™ FLEX can be used to clone CHO cells when combined with an appropriate 2X concentrated cell culture medium. Aspects of the CHO cell semi-solid cloning protocol specific to ClonaCell™-FLEX are not discussed in this document. Please contact **techsupport@stemcell.com** for more information.

1.2 Additional Support

We strive to provide you the best possible support. If you have any questions about ClonaCell™ products, please feel free to contact your local STEMCELL Technologies representative. You can also contact STEMCELL Technologies' Product & Scientific Support team by email (**techsupport@stemcell.com**) or by phone (1-800-667-0322) with any technical questions.

2.0 Before Planning a Semi-Solid Cloning Protocol

2.1 Choosing a Semi-Solid Cloning Medium

Table 2. ClonaCell™ semi-solid media for CHO cells

PRODUCT	CD[1]	ACF[2]	COMPONENTS	EXAMPLE CELL TYPES (*cell lines in italics have been tested in-house*)	GENERAL NOTES	RECOMMENDATIONS FOR PRE-CLONING CULTURE CONDITIONS AND CULTUREWARE
ClonaCell™-TCS Medium			• Pre-screened FBS and BSA; supplements; methylcellulose • Contains L-glutamine	*CHO-S, CHO-K1, CHO-DG44* (Other cell lines: *HEK-293, BHK-1,* BaF/3, Molt-4, K562, Jurkat, Daudi, UT-7, FD-5, B16F-10)	• Serum-containing medium supports high cloning efficiency and robust colony formation for a variety of suspension-adapted cell lines, as well as some adherent cell lines that have not been adapted to suspension culture.	• Semi-solid medium should be used with non-tissue culture-treated dishes to prevent cell adherence.
ClonaCell™-CHO ACF Medium		✓	• Recombinant proteins and synthetic components; methylcellulose • Does not contain L-glutamine	*CHO-S, CHO-K1, CHO-DG44* (Other cell lines: HEK-293)	• Defined, ACF formulation does not contain hydrolysates or other undefined components. • Single-cell cloning efficiency of CHO cells is greater in this ACF medium than in chemically defined, protein-free media.	• Cells must be adapted to suspension culture prior to cloning. • For best results, cells should be adapted to a protein-free cell culture medium (eg. ClonaCell™-CHO CD Liquid Medium) or a serum-free, animal component-free medium prior to cloning. • Semi-solid medium should be used with non-tissue culture-treated dishes to prevent cell adherence.
ClonaCell™-CHO CD Medium	✓	✓	• Synthetic components; methylcellulose • Does not contain L-glutamine	*CHO-S, CHO-K1,* proprietary CHO cell lines	• Protein-free formulation contains only synthetic components. • Generally supports lower cloning efficiencies than ClonaCell™-CHO ACF Medium, in particular when cells are plated at low cell densities. • Should only be used when a protein-free medium is required for the cloning stage.	• Cells must be adapted to suspension culture in a protein-free cell culture medium (eg. ClonaCell™-CHO CD Liquid Medium) prior to cloning. • Semi-solid medium should be used with non-tissue culture-treated dishes to prevent cell adherence.
ClonaCell™ FLEX	✓	✓	• Methylcellulose • Does not contain L-glutamine	*CHO-S,* CHO-K1, CHO-DG44 (Other cell lines: HEK-293, mouse hybridoma, human hybridoma, wide variety of suspension culture-adapted mammalian cells)	• Good option for developing a cloning protocol for cell lines that require specialized media formulations (e.g. CHO-DG44).	• ClonaCell™ FLEX is not a complete medium and must be combined with a 2X concentrated solution of a liquid cell culture medium. • Semi-solid medium should be used with non-tissue culture-treated dishes to prevent cell adherence.

1CD = Chemically defined and protein-free; 2ACF = Animal component-free

2.2 Subcloning Recommended for Initial Experiment

We recommend testing the semi-solid cloning procedure using your parental cell line or a previously established transfected cell line prior to performing a new transfection experiment. This will establish the suitability of the method and medium for your cells and establish optimal conditions for plating and culturing the cells.

If you plan to test the semi-solid cloning procedure in a transfection experiment without first performing a test with a parental or established transfected cell line, we recommend that you split the cells following transfection. Culture one portion of the cells using your current cloning medium and protocol and use the remaining portion to test the semi-solid cloning protocol. This will control for transfection efficiency as a variable in the experiment.

2.3 Upstream Cell Culture Media Compatibility

It is important that the cells are adapted to grow in an appropriate medium prior to semi-solid cloning. Cells may not survive if they are abruptly transferred from a richer expansion medium into an animal component-free or chemically defined medium for semi-solid cloning, as single-cell cloning imposes stresses that can restrict growth. Such stresses include physical separation from other cells and selective conditions.

See **Table 2** for recommended pre-cloning culture conditions.

See **Appendix A** for protocols for adapting cells to protein-free ClonaCell™-CHO CD Liquid Medium.

3.0 Equipment and Materials

3.1 Cultureware

Semi-solid cloning should be performed using **non-tissue culture-treated** cultureware. This prevents cells from adhering to the plastic and allows them to form colonies that are suspended in the semi-solid medium.

A variety of culture formats, such as 100 mm culture dishes (eg. Catalog #27110), 6-well plates or 96-well plates, can be used for semi-solid cloning (**Table 3**), depending on your desired protocol. The standard protocol outlined in the PISs uses 100 mm culture dishes. The testing protocol outlined in **Section 5** of this document uses 6-well plates.

Table 3. Recommended plating volumes for different cultureware

CULTURE FORMAT	SEMI-SOLID CELL SUSPENSION PLATED PER WELL
100 mm culture dishes	9.5 mL
6-well plates	1.5 mL
96-well plates	60 - 80 µL

Note on Plating Volumes

It is important to plate an appropriate volume of semi-solid cell suspension for the cultureware you are using. If the layer of medium is too thick in the plate or dish, picking individual colonies can be difficult. If the medium is too thin, the culture will dehydrate easily.

3.2 Needles and Syringes

Semi-solid medium **cannot be accurately transferred using a pipette**. Use an appropriately sized syringe (12 mL syringe for volumes between 4 mL and 10 mL; 5 mL syringe for volumes between 1 mL and 4 mL, 3 mL syringe for volumes between 0.5 mL and 1.5 mL) and blunt-end needle (Catalog #28110) to dispense semi-solid media.

Note on Needles

We recommend using blunt-end needles to transfer semi-solid media in order to prevent needle-stick injuries.

3.3 Incubator Conditions

Appropriate incubation conditions are crucial to allow cells to proliferate and grow into discrete colonies.

The incubator should be:

- Equipped with a full water pan to ensure the incubator is **well-humidified** and cultures are adequately hydrated.
- Maintained at **37°C and 5% CO_2 in air**.
- **Undisturbed** for 8 days, as movements due to nearby shakers or opening the incubator door repeatedly can prevent proper colony formation.

Note on Culture Hydration

Even cultures that appear well-hydrated to the eye can be under-hydrated, which restricts colony growth. Ensure incubator humidity remains high throughout the culture period. We recommend including a sterile water reservoir as part of your experimental set-up (see **Section 5.2, Step 4**) to ensure proper hydration of the cultures.

4.0 Storage of Semi-Solid Media

Upon delivery of ClonaCell™ semi-solid media to you laboratory, promptly place the bottle(s) at -20°C.

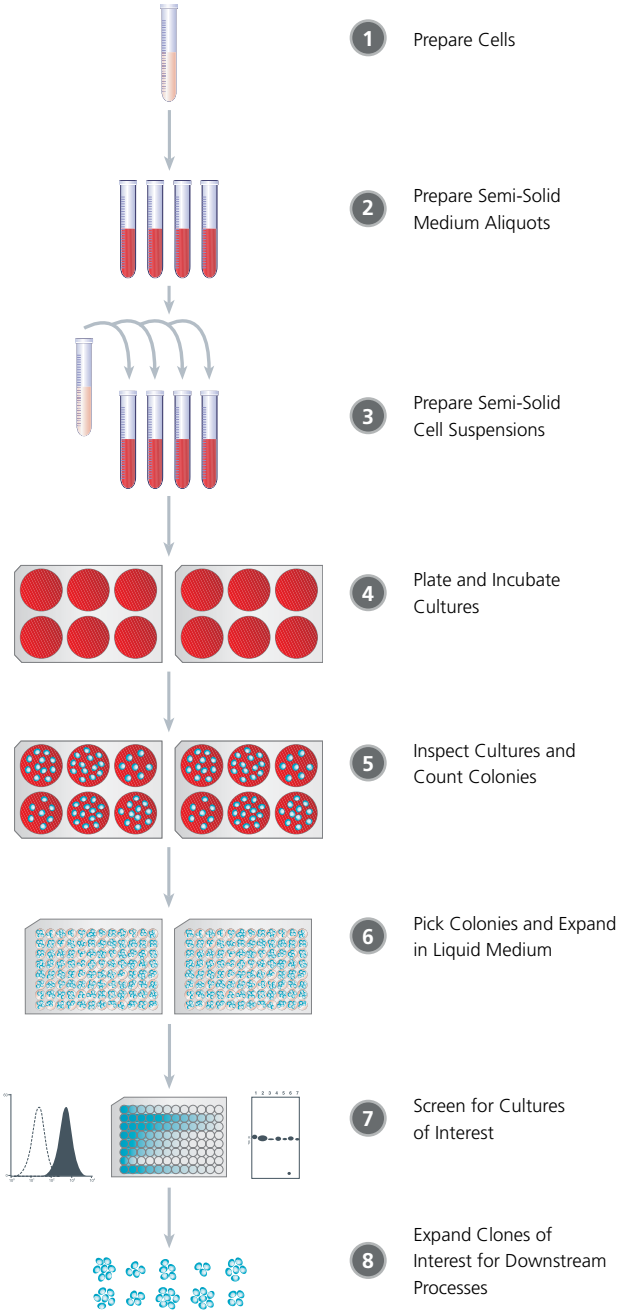
If the medium arrives completely thawed and is no longer cold to the touch, do not use the medium and contact our Technical Support department at **techsupport@stemcell.com**.

If the medium arrives partially thawed and is cold to the touch, place the bottle(s) at -20°C. There is no need to contact STEMCELL Technologies Technical Support if the medium is only partially thawed.

5.0 Testing Protocol (Parental or Previously Established CHO Cell Line)

As noted in **Section 2.2**, we recommend testing the semi-solid cloning procedure with either the parental cell line or a previously established transfected cell line to establish appropriate plating conditions. This section describes a **protocol to test four plating densities of a parental or previously established CHO cell line in triplicate**.

5.1 Protocol Overview (see Section 5.2 for details for each numbered step)



To Facilitate Fast Optimization of the Protocol:

KEEP DETAILED RECORDS OF THE PROCEDURES AND RESULTS. For example, cell counts and viability estimates, order in which test conditions are plated, incubation times, colony counts, qualitative observations of colonies, deviations from planned protocol, etc.

TAKE PHOTOS OF CULTURES BEFORE AND AFTER INCUBATION.

Note

Steps 6-8 may not be required when evaluating protocol with a parental or previously established CHO cell line.

5.2 Step-by-Step Testing Protocol

1 Prepare Cells

Prior to cloning in semi-solid medium, cells should be growing in liquid culture medium that is compatible with the semi-solid medium you will be using (see **Table 2 and Section 2.3**). See **Appendix A** for protocols for adapting cells to grow in ClonaCell™-CHO CD Liquid Medium.

Cells should be in **logarithmic growth phase** and have **> 90% viability** prior to initiating semi-solid cloning.

2 Prepare Semi-Solid Medium Aliquots

i) Thaw medium: The ClonaCell™ semi-solid medium should be thawed overnight at 2 - 8°C. **Do not thaw medium in a warm water bath**, as thawing too rapidly can cause clumping or other irregularities in the methylcellulose.

ii) Aliquot medium: This protocol recommends testing four plating concentrations for the semi-solid cloning procedure in triplicate in 6-well plates.

Aliquot semi-solid medium into four 50 mL conical tubes (5.4 mL per tube for ClonaCell™-CHO CD Medium and ClonaCell™-CHO ACF Medium; 4.8 mL per tube for ClonaCell™-TCS Medium; see **Table 4**). Label the tubes plating densities 1 - 4.

Each bottle of ClonaCell™ semi-solid medium can be used to prepare ~100 mL of cell suspension to be plated. This testing protocol uses ~24 mL of ClonaCell™-CHO CD Medium or ClonaCell™-CHO ACF Medium, or ~22 mL of ClonaCell™-TCS Medium. The remaining medium can be aliquoted and frozen for later use. These aliquots should be refrozen promptly after thawing, before cells are added to the semi-solid medium. Aliquot appropriately to avoid repeated freeze-thaw cycles.

iii) Warm medium aliquots: Pre-warm the aliquoted medium in a 37°C incubator for 15 - 30 minutes. **Do not warm medium in a warm water bath**, as warming too rapidly can cause clumping of the methylcellulose.

iv) Supplement medium aliquots: Prepare a 20X additive solution of any required supplements (eg. 4 - 8 mM L-glutamine;

Catalog #07100) in liquid cell culture medium (eg. ClonaCell™-CHO CD Liquid Medium, Catalog #03817).

Add 300 µL additive solution to each of the 4 tubes of aliquoted semi-solid medium. If using ClonaCell™-TCS Medium (Catalog #03814), add an additional 600 µL liquid medium to each aliquot. Mix the medium thoroughly by shaking vigorously. Let the medium stand to allow the bubbles to rise to the top and the viscous medium to collect at the bottom of the tube.

Note on Aliquoting Medium

Semi-solid medium should be transferred using a syringe and blunt-end needle, not a pipette.

Table 4. Component volumes for aliquots to test four plating densities in triplicate

	ClonaCell™-CHO ACF Medium; ClonaCell™-CHO CD Medium	ClonaCell™-TCS
Volume semi-solid medium provided in each bottle	90 mL	80 mL
Required semi-solid medium for triplicate data of 4 plating densities	24 mL	22 mL
Volume per test condition aliquot	6.0 mL (total)	6.0 mL (total)
• Semi-solid medium	***5.4 mL***	***4.8 mL***
• Additive solution[1]	***0.3 mL***	***0.3 mL***
• 20X concentrated cell suspension	***0.3 mL***	***0.3 mL***
• Liquid medium	***0.0 mL***	***0.6 mL***

1 20X solution of any required supplements in liquid cell culture medium. See Section 5.2 Step 2 iv.

3 Prepare Semi-Solid Cell Suspensions

Due to the great variation in growth rates and plating efficiencies between specific CHO cell lines, we recommend that you test a range of plating densities. This will enable determination of the appropriate concentration of cells required to achieve an optimal colony density for cloning (**Table 5**).

Table 5. Optimal colony densities for different cultureware

CULTURE FORMAT	COLONIES PER WELL
100 mm culture dishes	50 - 200
6-well plates (**used in this protocol**)	10 - 25
96-well plates	1

Note on Colony Densities

Having too many colonies per well makes it difficult to pick colonies and reduces the probability of monoclonality of the expanded cultures. Having too few colonies per well increases the amount of resources required to isolate a given number of cultures.

i) Choose a range of plating densities to test:

Method 1 for determining plating density range

If you have performed limiting dilution cloning experiments with the cell line you are using, you can use the number of cells plated per well and the number of growth-positive wells per 96-well plate typically found in these experiments to estimate an appropriate number of cells to plate per mL in semi-solid medium (**Equation 1**).

Equation 1.

$$\frac{\text{(number of cells plated per well)} \times 960}{\text{(number of wells positive for growth per plate)}} = \text{number of cells to plate per 1 mL semi-solid culture}$$

This type of estimate is only valid if cloning experiments in liquid medium were performed in a protein-free medium (for ClonaCell™-CHO CD Medium or ClonaCell™-CHO ACF Medium), or a defined medium containing recombinant proteins (for ClonaCell™-CHO ACF Medium only), as cloning efficiencies in these media are typically lower than in serum-containing media. This estimate is valid for ClonaCell™-TCS Medium if previous cloning experiments were performed in serum-containing liquid medium.

Suggested plating densities calculated using Equation 1 are provided in **Table 6**. We recommend testing four plating densities: 0.25X, 1X, 5X and 10X the estimated plating density determined using Method 1.

Table 6. Suggested plating densities (white boxes; cells/mL semi-solid cell suspension plated) based on example limiting dilution cloning results.

	# CELLS PLATED PER WELL (LIMITING DILUTION)					
# GROWTH-POSITIVE WELLS PER PLATE (LIMITING DILUTION)	1	10	50	100	500	1000
5	192	1,920	9,600	19,200	96,000	192,000
10	96	960	4,800	9,600	48,000	96,000
20	48	480	2,400	4,800	24,000	48,000
30	32	320	1,600	3,200	16,000	32,000
50	19	192	960	1,920	9,600	19,200

Method 2 for determining plating density range

If you have not previously performed limiting dilution cloning experiments with the cell line you are using, we recommend testing the four plating densities listed in **Table 7** that correspond to the medium you are testing.

Table 7. Recommended cell densities for cloning parental or previously established transfected cell lines.

PLATING DENSITY TEST CONDITION	PLATING DENSITY (CELLS/ML SEMI-SOLID CELL SUSPENSION PLATED)		
	ClonaCell™-CHO ACF Medium	ClonaCell™-CHO CD Medium	ClonaCell™ TCS Medium
1	20	200	20
2	50	500	50
3	100	1000	100
4	500	2000	200

ii) Count viable cells: Count viable CHO cells using Trypan Blue staining with a hemocytometer or using an automated cell counter.

iii) Prepare cell dilutions: Prepare 20X concentrated cell suspensions for each of the four cell concentrations you will be testing by diluting the cells in liquid cell culture medium (eg. ClonaCell™-CHO CD Liquid Medium, Catalog #03817). Label the concentrated cell suspensions as plating densities 1 - 4.

iv) Add cells to semi-solid medium: Add 300 µL of each concentrated cell suspension to the corresponding aliquot of supplemented semi-solid medium.

Replace the cap and shake the semi-solid cell suspension vigorously for 1 - 2 minutes. The medium should appear opaque. Let the medium sit for 15 minutes to allow the medium to pool at the bottom of the tube and the bubbles to rise to the surface.

4 Plate and Incubate Cultures

i) Plate the semi-solid cell suspensions: Use a blunt-end 16-gauge needle attached to a 5 mL disposable syringe to plate (in triplicate) 1.5 mL semi-solid cell suspension for each of the four plating densities into individual wells of non-tissue culture-treated 6-well plates.

Tilt plates gently and rotate to ensure that the medium is evenly distributed over the bottom of the wells.

ii) Incubate the cultures: Place the covered 6-well plates inside a large, covered dish (eg. Catalog #38039) containing an uncovered 100 mm dish of sterile water. Alternatively, sterile water can be added to half-fill the space between wells before the 6-well plates are covered.

Note on Incubation Conditions

It is important for colony growth that cultures

- are well-humidified throughout the culture period
- are not disturbed during the first eight days of incubation (do not check on colonies before this period)

5 Inspect Cultures and Count Colonies

After 10 - 14 days, depending on the doubling time of your cell line, inspect the cultures for the presence of colonies. Colonies should be visible to the naked eye, typically 0.5 - 1.0 mm in diameter.

Count the colonies that have grown in the wells corresponding to each plating density. Compare these values against the optimal colony densities in **Table 5**. Take photos of the plates (including the culture conditions used for each plate) to keep a record of colony morphology and number.

Note on Incubation Time

A longer incubation time (e.g. additional 7 days) may be required for some cell lines or selection systems.

Steps 6-8 may not be required when evaluating the protocol with a parental or previously established CHO cell line.

6 Pick Colonies and Expand in Liquid Medium

i) Prepare 96-well plates: Add 200 µL liquid culture medium (containing any supplements required for cell expansion) into each well of a 96-well plate.

ii) Pick colonies: Set a pipette to 10 µL and aspirate the colonies individually, using a new, sterile pipette tip for each colony. Transfer each colony to the liquid medium in a new well of the 96-well plate. Gently resuspend each colony by pipetting up and down several times in the liquid medium. A single-cell suspension is not required but gently dispersing the cells will promote proliferation.

iii) Incubate the cultures: Incubate the liquid cultures in a well-humidified incubator at 37°C and 5% CO_2 for 3 - 4 days.

7 Screen for Cultures of Interest Using an Appropriate Assay

8 Expand Clones of Interest for Downstream Processes

Cultures that test positive during screening may be expanded for further analysis, subcloning or cryopreservation. Subcloning is recommended for generating stable, high-expressing clones.

6.0 Testing Protocol (Freshly Transfected CHO Cells)

The semi-solid cloning protocol for newly transfected cells is essentially the same as the protocol outlined in **Section 5.2** with the following changes and additional considerations:

1. Antibiotic/selection agent concentration
2. Transfection
3. Higher plating densities

When testing the semi-solid cloning protocol on newly-transfected cells for the first time, we recommend that you culture a portion of the transfected cells using your current cloning medium and protocol to control for transfection efficiency as a variable in the experiment.

6.1 Antibiotic/Selection Agent Concentration

Your cells may grow differently in the presence of an antibiotic or selection agent in semi-solid medium than in liquid medium. The appropriate concentration of selection agents should be determined prior to plating freshly transfected CHO cells in selective semi-solid medium.

To determine an appropriate concentration of selection agents, plate the parental cell line in semi-solid medium containing a range of concentrations of selection agents from 0.25X to 2X the concentration you would use for the same cells in liquid medium. Use the semi-solid cloning technique described in **Section 5.2** and plate cells at the optimal plating density for the parental CHO cell line.

Inspect the plates after 10 - 12 days of culture. Select the lowest concentration of selection agent for which there is no growth of the non-transfected parental CHO cell line for further experiments.

6.2 Transfection

Prior to transfection, cells should be in logarithmic growth phase and have > 90% viability. Transfect the cells using your normal protocol. Use the same protocol for post-transfection recovery as you would when cloning in liquid medium.

6.3 Plating density

Freshly transfected cells need to be plated at a higher plating density than the parental cell line or a previously established transfected cell line to achieve an optimal colony density.

We recommend testing a range of plating densities to determine the optimal cell concentration for plating. This will enable you to determine an appropriate plating density, and exclude plating densities that result in too many or too few colonies per dish (see **Table 5** for optimal colony densities).

A Freshly Transfected Cell Pool:

- Tends to have lower viability than the parental cell line
- Is not likely to be in the logarithmic growth phase
- Is likely to contain a significant proportion of cells that have not been stably transfected
- Is subjected to chemical or nutritional selective pressure

Choose a range of plating densities to test:

Method 1 for determining plating density range

If you have performed limiting dilution cloning experiments with transfectants of the cell line and transfection method you are using, you can use the number of cells plated per well and the number of growth-positive wells per 96-well plate typically found in these experiments to estimate an appropriate number of cells to plate per mL in semi-solid medium (see **Equation 1** and **Table 6** in **Section 5.2 Step 3 i** for details).

Method 2 for determining plating density range

If you have not previously performed limiting dilution cloning experiments with transfectants of the particular cell line or transfection method you are using, we recommend testing the four plating densities listed in **Table 8** that correspond to the medium you are testing.

Table 8. Recommended cell densities for cloning freshly transfected cells.

PLATING DENSITY TEST CONDITION	PLATING DENSITY (CELLS/ML SEMI-SOLID CELL SUSPENSION PLATED)		
	ClonaCell™-CHO ACF Medium	ClonaCell™-CHO CD Medium	ClonaCell™ TCS Medium
1	2,500	2,500	2,500
2	25,000	25,000	25,000
3	100,000	100,000	100,000
4	250,000	250,000	250,000

Appendix A: Adaptation of CHO Cells to Protein-Free ClonaCell™-CHO CD Liquid Medium (Catalog #03817)

ClonaCell™-CHO CD Liquid Medium does not contain L-glutamine. Supplement with 4 - 8 mM L-glutamine if needed. Additional supplements, antibiotics or selection agents may also be added to the medium, depending on the cell line and application.

The viability of cells should be greater than 90% and the cells should be in logarithmic growth phase prior to adapting directly or gradually in ClonaCell™-CHO CD Liquid Medium.

Note on Medium Supplementation

When adapting cells for cloning in ClonaCell™-CHO ACF Medium or ClonaCell™-TCS Medium, it may be advantageous to use ClonaCell™-CHO ACF Supplement (Catalog #03820) during adaptation and culture prior to cloning, in order to increase the growth of cells at low cell density.
See **www.clonacell.com** for more information on ClonaCell™-CHO ACF Supplement.

Method 1. Direct Adaptation of CHO cells to ClonaCell™-CHO CD Liquid Medium

Most cells can be transferred directly into ClonaCell™-CHO CD Liquid Medium.

1. Determine the concentration and viability of the cells in culture.
2. Seed culture flasks at 3 x 10^5 viable cells/mL.
3. Incubate at 37°C in a humidified incubator at 5% CO_2.
4. Passage every 3 - 4 days at seeding densities of 1 - 3 x 10^5 viable cells/mL. Passage for a minimum of 3 passages where viability is > 90% and cell growth is robust to ensure that cells have adapted successfully prior to subcloning or transfection.

Method 2. Gradual Adaptation of CHO cells to ClonaCell™-CHO CD Liquid Medium

Gradual adaptation of CHO cells may be required if direct adaptation results in low viability or poor growth.

1. Culture cells at 3 x 10^5 cells/mL in a 3:1 mixture of the original growth medium and ClonaCell™-CHO CD Liquid Medium.
2. After 3 - 4 days, determine the cell concentration and viability of the culture. If viability is > 90% and cell concentration is > 1 x 10^6 cells/mL, proceed to the next step. If viability is low and growth is slow, maintain or passage the cells in the current media mixture until viability and growth increase.
3. Culture cells at 3 x 10^5 cells/mL in a 1:1 mixture of the original medium and ClonaCell™-CHO CD Liquid Medium.
4. After 3 - 4 days, examine cells as in Step 2.
5. Culture cells at 3 x 10^5 cells/mL in a 1:3 mixture of the original medium and ClonaCell™-CHO CD Liquid Medium.
6. Examine cells as in Step 2.
7. Culture cells at 3 x 10^5 cells/mL in 100% ClonaCell™-CHO CD Liquid Medium. Examine cells as in Step 2.
8. Passage every 3 - 4 days at seeding densities of 1 - 3 x 10^5 viable cells/mL. Passage for a minimum of 3 passages to ensure that viability is > 90% and cell growth is robust prior to subcloning or transfection.

Appendix B: Optimizing Viscosity of Semi-Solid Media

Most CHO cell lines should grow well under the standard viscosity conditions described in **Section 5.2**. In some cases (**Table I**), however, it may be beneficial to test a range of medium viscosities. This is easily accomplished by varying the total volume of liquid added to ClonaCell™ semi-solid medium.

Table I. Effects of viscosity on semi-solid cultures

CONDITION OF MEDIUM	POTENTIAL RESULT	POTENTIAL CAUSES
Viscosity too low (runny)	• Smeared or "blurry" colonies • Indistinct colonies	• Too much liquid added to semi-solid medium • High atmospheric humidity • High incubator humidity • Incubator temperature too low • Plate disturbed during early incubation • Some brands of plastic cultureware
Viscosity too high (gelatinous)	• Poor colony growth • No colonies	• Too little liquid added to semi-solid medium • Dry atmospheric conditions • Low incubator humidity • Incubator temperature too high (fast evaporation)

The recommended volumes in this appendix are expressed as the volumes of semi-solid and liquid media required per 1 mL semi-solid cell suspension plated. See **Table 3** for recommended plating volumes for 100 mm dishes and 6-well plates. We recommend preparing at least 10% more semi-solid cell suspension than you plan to plate, as it is not possible to transfer all of the medium for plating.

Note on Liquid Volumes

The total volume of liquid added to the semi-solid medium includes any additives, selection agents and cells.

Table II. Volumes of semi-solid and liquid media for viscosity optimization of ClonaCell™-CHO CD Medium and ClonaCell™-CHO ACF Medium (90 mL semi-solid medium provided in each bottle)

VOLUME SEMI-SOLID MEDIUM (PER ML PLATED CULTURE)	TOTAL VOLUME OF LIQUID (PER ML PLATED CULTURE)
850 µL	150 µL
900 µL	100 µL
950 µL	50 µL
970 µL	30 µL

Table III. Volumes of semi-solid and liquid media for viscosity optimization of ClonaCell™-TCS Medium (80 mL semi-solid medium provided in each bottle)

VOLUME SEMI-SOLID MEDIUM (PER ML PLATED CULTURE)	TOTAL VOLUME OF LIQUID (PER ML PLATED CULTURE)
750 µL	250 µL
800 µL	200 µL
850 µL	150 µL
900 µL	100 µL

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