Protocol

CloneMatrix

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Introduction

CloneMatrix is a unique semi-solid concentrate designed to allow conversion of liquid media into semi-solid form, thus facilitating the semi-solid cloning process.

Semi-solid media may be prepared from *CloneMatrix* to suit a wide range of cell types including hybridomas, myelomas, adherent CHO and HEK, serum-free CHO and HEK, and embryonic stem cells.

Developed specifically for optimised use with Genetix' *ClonePix* mammalian colony picking systems, *CloneMatrix*-based semi-solid media support colony formation as well as fluorescent assays using *CloneDetect Agent*.

Supplied as 40ml in 100ml bottles, concentrated media, supplements and selective agents can be added for optimal growth of your cells without the need to aliquot.

CloneMatrix is free from animal sourced products and suitable for users wishing to work under chemically-defined conditions.

Storage instructions

Store ***CloneMatrix*** at -20°C.

Should *CloneMatrix* be partially defrosted upon receipt, please re-freeze prior to use. Thaw *CloneMatrix* at 4°C over night. Do not shake contents until completely defrosted. Allow to adjust to room temperature prior to use. Defrosted *CloneMatrix* is stable at 4°C for up to 6 months.

CloneMatrix-CHO is supplied with one 2ml aliquot of ***CloneXL Reagent*** per 100ml bottle. Store *CloneXL Reagent* at -20°C. Thaw *CloneXL Reagent* at 4°C over night or at room temperature just prior to use. Allow to adjust to room temperature prior to use.

Semi-solid media prepared from *CloneMatrix* should be used as soon as possible. Once prepared, semi-solid media may be stored at 4°C for up to one week.

For research use only.

Protocol

Please note;
The recommended plating procedures differ depending on the cell type used.

Follow general instructions for **Media Preparation** (Part 1) first and then select and follow the appropriate instructions for **Cell Plating** (Part 2).

1. MEDIA PREPARATION

Semi-solid media prepared from one bottle (40ml) of *CloneMatrix* is sufficient for one of the following;

- up to eight Genetix PetriWell-6 Plates (plate 2ml of media per well)
- up to ten Genetix PetriWell-1 Plates or 100mm tissue culture dishes (plate 9ml of media each).

Prior to use, thaw *CloneMatrix* at 4°C overnight. Do not shake contents until completely defrosted. Allow to adjust to room temperature prior to use (do not pre-warm to 37°C). **Prepare media from components adjusted to room temperature, only.**

For 100ml of semi-solid media add the following to *CloneMatrix* (40ml):

- 50ml of liquid media of choice (2x concentrated)
- 10ml of additional components (e.g. growth additives such as *CloneXL Reagent* or serum, selection agent etc.)

Bring up to a final volume of 100ml with sterile tissue culture water if necessary.

If volume of additional components exceeds 10ml, ensure the final volume of the semi-solid media does not exceed 110ml.

If using *CloneMatrix-CHO*, add 2ml of *CloneXL Reagent* to the semi-solid media at this stage, as part of the 100ml final volume.

Shake bottle vigorously for ca. 10 seconds to mix the components thoroughly. Allow time for bubbles to escape (Ca. 10 min at room temperature). Any bubbles remaining after this will dissipate during the plate incubation.

For fluorescent detection assays, *CloneDetect Agent* may be added at this stage (see list of *CloneDetect Agent* products included). Mix *CloneDetect* gently into the prepared media and protect bottle from direct light (please refer to the *CloneDetect* protocol for detailed instructions).

Next: Please select and follow **plating instructions applicable for your cell type** from the sections below.

2. INSTRUCTIONS FOR CELL PLATING

2.1 Hybridoma

Hybridoma cells growing in semi-solid conditions form discreet, spherical colonies suspended in the semi-solid media. The figure below shows images of typical hybridoma colonies in semi-solid culture.

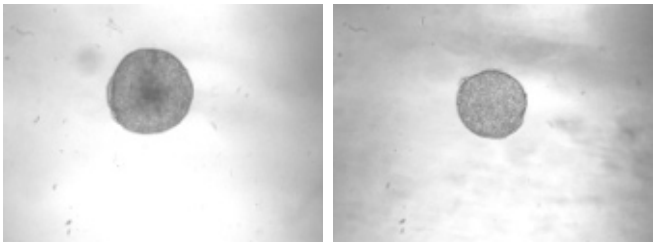


Figure 1. Images of hybridoma colonies after 7 days of growth in semi-solid media prepared using *CloneMatrix* (bright field, 100x magnified).

The correct density of colonies in the culture dish is key to the success of colony selection and the automated picking process (25-50 colonies/ml of semi-solid media are recommended as optimal). It is therefore crucial to thoroughly **optimise the seeding densities**.

Seeding densities required to achieve an optimal density of colonies depend on the inherent plating efficiency of the cells used as well as the viability and growth phase of the cell suspension culture at the time of plating.

The following ranges of seeding densities may serve as a guide line for your optimisation;

- Stable and robust hybridoma cell lines
  50 cells/ml, 100 cells/ml, 150 cells/ml
- Fresh fusions
  1×10^4 cells/ml to 1×10^6 cells/ml

Please note;
When plating fresh hybridoma fusions the optimal seeding density is highly dependent on the fusion efficiency as well as the kinetics and efficiency of selection. Cells may require a period of recovery prior to seeding in semi-solid media. For the successful selection of hybrids, the selection pressure applied (i.e. concentration of HAT) may also require optimisation.

Prepare a semi-solid media from *CloneMatrix* and a 2x concentrated liquid hybridoma media as described in section 1 above and supplement with at least 10% serum.

Ideally, the liquid media concentrate used for the preparation of a semi-solid media using *CloneMatrix* should be consistent with the routinely used liquid culture media. A period of adaptation of cells to the subsequently used media may otherwise be necessary.

Based on the density and viability of the cell suspension culture as determined e.g. by haemocytometer count, add the correct amount of cells (ideally in less than 100µl culture volume) to the semi-solid media. Invert the tube several times to gently distribute the cells evenly throughout the semi-solid media.

To plate out;
Dispense ca. 2ml per well of a Genetix PetriWell-6 Plate and 9ml of media for every Genetix PetriWell-1 Plate or 100mm tissue culture dish. This may be achieved using a 10ml pipette or by pouring.

Tilt the culture dish gently to ensure even distribution.

Place in an incubator at 37°C, 5% CO_2 for 5-10 days to allow colonies to grow.

Leave undisturbed for at least 4 days.

Verify the presence of growing colonies at a suitable time point using a light microscope.

2.2 Myeloma

Myeloma cells growing in semi-solid conditions form discreet, spherical colonies suspended in the semi-solid media. Figure 2 below shows images of typical myeloma colonies in semi-solid culture.

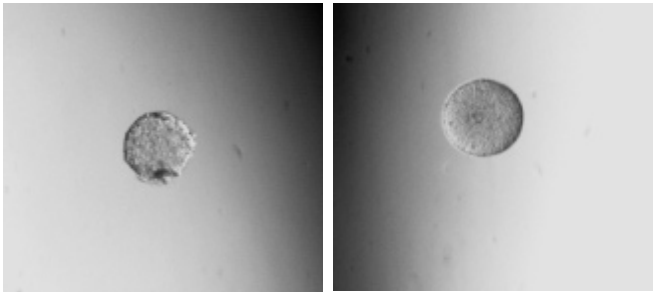


Figure 2. Images of myeloma colonies after 8 days of growth in semi-solid media prepared using *CloneMatrix* (bright field, 100x magnified).

The correct density of colonies in the culture dish is key to the success of colony selection and the automated picking process (25-50 colonies/ml of semi-solid media are recommended as optimal). It is therefore crucial to thoroughly **optimise the seeding densities**.

Seeding densities required to achieve an optimal density of colonies depend on the inherent plating efficiency of the cells used as well as the viability and growth phase of the cell suspension culture at the time of plating.

The following ranges of seeding densities may serve as a guide line for your optimisation;

- Stable and robust myeloma cell lines
  50 cells/ml, 100 cells/ml, 150 cells/ml
- Fresh transfections
  $1x10^3$ cells/ml, $2x10^3$ cells/ml, $5x10^3$ cells/ml, $1x10^4$ cells/ml

Please note;
When plating fresh myeloma transfections the optimal seeding density is highly dependent on the transfection efficiency as well as the kinetics and efficiency of selection. Cells may require a period of recovery prior to seeding in semi-solid media. For the successful selection of transfectants, the selection pressure applied may also require optimisation.

Prepare a semi-solid media from *CloneMatrix* and a 2x concentrated liquid culture media as described in section 1 above and supplement with at least 10% serum.

Ideally, the liquid media concentrate used for the preparation of a semi-solid media using *CloneMatrix* should be consistent with the routinely used liquid culture media. A period of adaptation of cells to the subsequently used media may otherwise be necessary.

Based on the density and viability of the cell suspension culture as determined e.g. by haemocytometer count, add the correct amount of cells (ideally in less than 100µl culture volume) to the semi-solid media. Invert the tube several times to gently distribute the cells evenly throughout the semi-solid media.

To plate out;
Dispense ca. 2ml per well of a Genetix PetriWell-6 Plate and 9ml of media for every Genetix PetriWell-1 Plate or 100mm tissue culture dish. This may be achieved using a 10ml pipette or by pouring.

Tilt the culture dish gently to ensure even distribution.

Place in an incubator at 37°C, 5% CO_2 for 5-10 days to allow colonies to grow.

Leave undisturbed for at least 4 days.

Verify the presence of growing colonies at a suitable time point using a light microscope.

2.3 CHO-S (serum-free, suspension-adapted)

Suspension-adapted CHO cells growing in semi-solid conditions form discreet, spherical colonies suspended in the semi-solid media (see example images below).

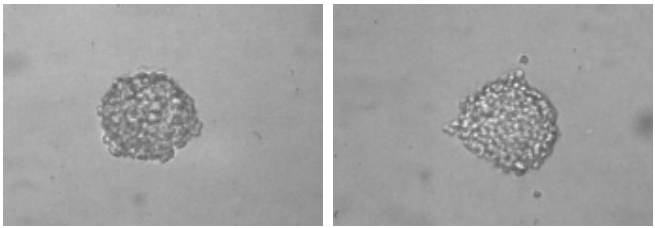


Figure 3: Images of colonies of CHO-S cells grown in serum-free, semi-solid media prepared using *CloneMatrix-CHO*. Images were taken on day 10 post-plating using a white light microscope at 100x magnification.

The correct density of colonies in the culture dish is key to the success of colony selection and the automated picking process (25-50 colonies/ml of semi-solid media are recommended as optimal). It is therefore crucial to thoroughly **optimise the seeding densities**.

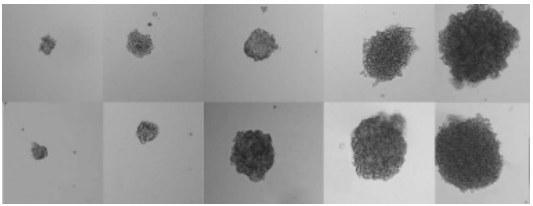
Seeding densities required to achieve an optimal density of colonies depend on the inherent plating efficiency of the cells used as well as the viability and growth phase of the cell suspension culture at the time of plating.

The following ranges of seeding densities may serve as a guide line for your optimisation;

- Stable and robust CHO-S cell lines (e.g. parental CHO-S cell line)
  250 cells/ml, 500 cells/ml, 1000 cells/ml
- Other CHO-S lines (e.g. non-clonal expressing cell line)
  500 cells/ml, 1000 cells/ml, 2000 cells/ml
- Fresh transfections
  $1x10^3$ cells/ml, $2x10^3$ cells/ml, $5x10^3$ cells/ml, $1x10^4$ cells/ml

Please note;
When plating fresh transfections the optimal seeding density is highly dependent on the transfection efficiency as well as the kinetics and efficiency of selection. Cells may require a period of recovery prior to seeding in semi-solid media. The selection pressure applied may also require optimisation.

Well established CHO-S cells in optimal semi-solid culture conditions will, on average, undergo one division in every 24 hours. Figure 4 below shows examples of colony sizes as observed in a semi-solid culture system.



Estimates:

Cells (seen)	~58	88-97	n.a.	n.a	n.a.
Cells/colony	125	256-512	1024-2048	2048-4096	8196
Cell divisions	7	8-9	10-11	11-12	13
Days growth	8	10	12	13	14

Figure 4: Growth progression of CHO-S colonies in semi-solid media. Images of representative colonies captured by bright field microscopy (100x magnification) and are arranged by colony age and size. (n.a. not applicable).

Prepare a semi-solid media from *CloneMatrix* or *CloneMatrix-CHO* and a 2x concentrated liquid media for serum-free culture of CHO as described in section 1 above.

Ideally, the liquid media concentrate (at least 2x) used should be consistent with the routinely used liquid culture media. A period of adaptation of cells to the subsequently used media may otherwise be necessary.

The *CloneXL Reagent* supplied with *CloneMatrix-CHO* is an animal-free, chemically-defined growth supplement specifically developed to enhance the growth of suspension-adapted CHO in animal free media. Please add the whole 2ml aliquot per bottle of *CloneMatrix* as part of the 100ml final volume.

Alternatively, you may chose to supplement with up to 10% conditioned media to improve plating efficiency and colony growth. However, the composition and quality of conditioned media is highly dependent on its source and method of preparation.

Based on the density and viability of the cell suspension culture as determined e.g. by haemocytometer count, add the correct amount of cells (ideally in less than 100µl culture volume) to the semi-solid media. Invert the tube several times to gently distribute the cells evenly throughout the semi-solid media.

To plate out;
Dispense 2ml per well of a Genetix PetriWell-6 Plate or 9ml of media per Genetix PetriWell-1 Plate or 100mm tissue culture dish.

This may be achieved using a 10ml pipette or by pouring.

Tilt the culture dish gently to ensure even distribution.

The incubation time required for CHO-S in semi-solid media requires precautions to keep the semi-solid media well hydrated. To this end fill areas of the plate between wells with ca. 2ml of sterile water.

Place cultures in an incubator at 37°C, 5% CO_2 for 7-10 days to allow colonies to grow.

Leave undisturbed for at least 4 days.

Verify the presence of growing colonies at a suitable time point using a light microscope.

2.4 CHO adherent (in the presence of serum)

For adherent CHO lines, two different plating protocols have been developed for the growth of colonies in semi-solid media;

2.4a) CHO-plating for suspended colonies

2.4b) CHO-plating for adherent colonies

Genetix' *ClonePix* systems are capable of picking suspended colonies as well as adherent colonies growing at the bottom of the culture dish providing that they are discreet from one another (see Figure 5 below for typical images).

Protocol a) is the preferred method as suspended colonies offer the inherent advantage of being more compact, which allows clones to be plated and picked at a higher density with low risk of cross contamination.

However, not all adherent CHO lines are suitable for this approach.

Depending on the properties of the CHO line used, cells will more or less readily adhere to the bottom of the culture dish and form a layer of adherent cells in the background. For highly adherent CHO lines, protocol b) will be more suitable.

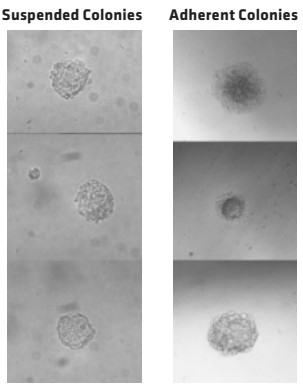


Figure 5: Images of CHO colonies in semi-solid media prepared using *CloneMatrix*. Cells grow as suspended colonies (left panel) when plated according to protocol a).
Adherent colonies (right panel) obtained by plating according to protocol b). Images were taken using a white light microscope at 100x magnification on day 10 post-plating.

2.4a) CHO-plating for suspended colonies

Prepare a semi-solid media from *CloneMatrix* and a 2x concentrated liquid media for culture of CHO as described in section 1 above and supplement with a small amount of serum (1% or less if possible).

The correct density of colonies in the culture dish is key to the success of colony selection and the automated picking process (25-50 colonies / ml of semi-solid media are recommended as optimal). It is therefore crucial to thoroughly **optimise the seeding densities**.

Seeding densities required to achieve an optimal density of colonies depend on the inherent plating efficiency of the cell line used, the viability and growth phase of the cell suspension culture at the time of plating, as well as the serum concentration in the media.

We recommend testing a range of seeding densities between 200 cells/ml and 500 cells/ml for stable and robust cell lines.

Based on the density and viability of the cell suspension culture as determined e.g. by haemocytometer count, add the correct amount of cells (ideally in less than 100µl culture volume) to the semi-solid media. Invert the tube several times to gently distribute the cells evenly throughout the semi-solid media.

Please note;
It is crucial that the appropriately treated culture plastics are used.
Plate into **non TC-treated** culture dishes.

To plate out;
Dispense 2ml per well of a Genetix PetriWell-6 Plate or 9ml of media per Genetix PetriWell-1 Plate or 100mm tissue culture dish. This may be achieved using a 10ml pipette or by pouring.

Tilt the culture dish gently to ensure even distribution.

Place in an incubator at 37°C, 5% CO_2 for 7-10 days to allow colonies to grow. Leave undisturbed for at least 4 days.

Verify the presence of growing colonies at a suitable time point using a light microscope.

2.4b) CHO-plating for adherent colonies

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 thoroughly **optimise the seeding densities**.

For picking on Genetix' *ClonePix* systems the optimal density of adherent CHO colonies is ca. 25 colonies per 1 ml of semi-solid media.

For the procedure described below plating efficiency is high. We recommend evaluation of a range of seeding densities (ca. 25 cells/ml and 50 cells/ml).

Please note;
It is crucial that the appropriately treated culture plastics are used.
Plate into **TC-treated** culture dishes in the usual liquid media supplemented with serum (as per standard protocol).

Plate cells in 2ml media per well of a Genetix PetriWell-6 Plate or 9ml of media per Genetix PetriWell-1 Plate or 100mm tissue culture dish.

Place in an incubator at 37°C, 5% CO_2 over night and allow cells to adhere.

Verify good adherence using a light microscope.

Prepare semi-solid media as described in section 1 above and supplement with the same amount of serum as used in routine liquid culture.

Carefully aspirate liquid media and gently overlay with semi-solid media immediately.

Use 2ml of semi-solid media per well of a Genetix PetriWell-6 Plate or 9ml of media per Genetix PetriWell-1 Plate or 100mm tissue culture dish.

Tilt the culture dish gently to ensure even distribution of the semi-solid media.

Place in an incubator at 37°C, 5% CO_2 for 4-10 days to allow colonies to grow. Leave undisturbed for at least 4 days.

Verify the presence of growing colonies at a suitable time point using a light microscope.

2.5 HEK293 (serum-free, suspension adapted)

For serum-free HEK293 cells two different plating protocols have been developed for the growth of colonies in semi-solid media;

2.5a) HEK293-plating for suspended colonies

2.5b) HEK293-plating for adherent colonies

Genetix' *ClonePix* systems are capable of picking suspended colonies as well as adherent colonies growing at the bottom of the culture dish providing that they are discreet from one another (for typical images see figure 6 below).

Suspended colonies tend to be more compact, which allows clones to be plated and picked at a higher density with low risk of cross contamination.

However, not all serum-free HEK293 lines may be suitable for this approach and sufficient cell growth may only be obtained under adherent conditions.

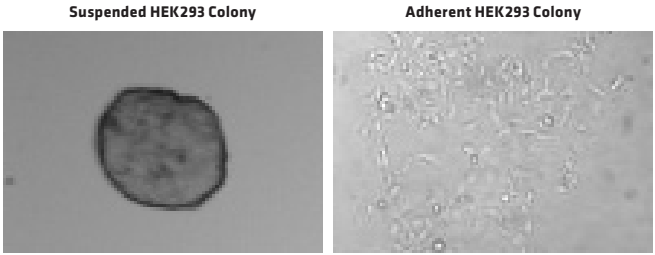


Figure 6: Images of HEK293 colonies in semi-solid media. Cells grown as suspended colonies (left panel, plating protocol a) and as adherent colonies (right panel, plating protocol b). Images were taken using a white light microscope at 100x magnification on day 10 post-plating.

To ensure the success of semi-solid culture of HEK293 cells, cultures need to be well established and maintained in a high density shaken culture routine. The methods described below have proven successful.

Maintenance of HEK293;

Culture cells in a suitable liquid culture media in a shaking incubator. Grow cultures up to a density of 1.5-1.7 x 10^6 cells/ml, splitting cultures once most weeks (very occasionally twice, depending on the cell count). Once maximum density has been reached, dilute cultures back to 3x10^5 cells/ml (equates to ca. 1 part of culture to 5 parts of fresh pre-warmed media).

Under this routine cultures should maintain viabilities of above 85% (averages around 95%).

Preparation of conditioned media;

The plating efficiency can be improved significantly by supplementation of the semi-solid media with 10% conditioned media.

The composition and quality of conditioned media is paramount to the success of the semi-solid culture procedure whilst being highly dependent on its source and method of preparation.

Culture cells as described above and schedule preparation of conditioned media around 24 to 36 hours after splitting to avoid accumulation of potentially detrimental waste products.

Remove required volume of cell culture (e.g. obtain 5ml of cell culture for a semi-solid plating experiment requiring one 6-well plate). Transfer culture volume into a 50ml sterile syringe fitted with a 0.2µm CAT disc filter (Lab3; 513-1903).

Filter culture through carefully and slowly. Use conditioned media immediately after preparation.

2.5a) HEK293-plating for suspended colonies

Maintain cultures as described above and schedule seeding in semi-solid media around 32 hours after splitting.

The supplementation of the semi-solid media with 10% conditioned media is strongly advised. Prepare fresh conditioned media in time for semi-solid plating (refer to method described above).

Prepare a semi-solid media from *CloneMatrix* and a 2x concentrated liquid media for serum-free culture of HEK293 as described in section 1 above.

The correct density of colonies in the culture dish is key to the success of colony selection and the automated picking process (25-50 colonies/ml of semi-solid media are recommended as optimal). It is therefore crucial to thoroughly **optimise the seeding densities**.

Seeding densities required to achieve an optimal density of colonies depend on the inherent plating efficiency of the cells used as well as the viability and growth phase of the liquid culture at the time of plating.

We recommend evaluation of a range of seeding densities (ca. 500 cells/ml to 1500 cells/ml).

Based on the density and viability of the cell suspension culture as determined e.g. by haemocytometer count, add the correct amount of cells (ideally in less than 100µl culture volume) to the semi-solid media. Invert the tube several times to gently distribute the cells evenly throughout the semi-solid media.

Please note;
It is crucial that the appropriately treated culture plastics are used. Plate into **non TC-treated** culture dishes in an appropriately prepared semi-solid media.

Plate cells in 2ml of semi-solid media per well of a Genetix PetriWell-6 Plate or 9ml of media per Genetix PetriWell-1 Plate or 100mm tissue culture dish.

Tilt the culture dish gently to ensure even distribution of the semi-solid media.

HEK 293 colonies take ca. 12 to 14 days to form colonies that can comfortably be picked (>50 cells/colony). This requires precautions to keep the semi-solid media well hydrated. To this end fill areas of the plate between wells with ca. 2ml of sterile water.

Place in an incubator at 37°C, 5% CO_2 to allow colonies to grow. Leave undisturbed for at least 4 days.

Verify the presence of growing colonies at a suitable time point using a light microscope.

2.5b) HEK293-plating for adherent colonies

Maintain cultures as described above and schedule seeding in semi-solid media around 32 hours after splitting.

The supplementation of the semi-solid media with 10% of conditioned media is strongly advised. Prepare fresh conditioned media in time for semi-solid plating (refer to method described above).

Prepare a semi-solid media from *CloneMatrix* and a 2x concentrated liquid media for serum-free culture of HEK293 as described in section 1 above.

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 thoroughly **optimise the seeding densities**.

For picking on Genetix, *ClonePix* systems the optimal density of adherent HEK colonies is ca. 15 colonies per 1ml of semi-solid media.

We recommend evaluation of a range of seeding densities (ca. 200 cells/ml to 1000 cells/ml).

Based on the density and viability of the cell suspension culture as determined e.g. by haemocytometer count, add the correct amount of cells (ideally in less than 100µl culture volume) to the semi-solid media. Invert the tube several times to gently distribute the cells evenly throughout the semi-solid media.

Please note;
It is crucial that the appropriately treated culture plastics are used. Plate into **TC-treated** culture dishes in an appropriately prepared semi-solid media.

Plate cells in 2ml of semi-solid media per well of a Genetix PetriWell-6 Plate or 9ml of media per Genetix PetriWell-1 Plate or 100mm tissue culture dish.

Tilt the culture dish gently to ensure even distribution of the semi-solid media.

HEK 293 colonies take ca. 9 to 14 days to form colonies that can comfortably be picked (>74 cells/colony). This requires precautions to keep the semi-solid media well hydrated. To this end fill areas of the plate between wells with ca. 2ml of sterile water.

Place in an incubator at 37°C, 5% CO_2 to allow colonies to grow. Leave undisturbed for at least 4 days.

Verify the presence of growing colonies at a suitable time point using a light microscope.

2.6 Mouse Embryonic Stem Cells

Mouse embryonic stem cells growing in semi-solid conditions form discreet colonies of a range of morphologies reflecting the degree of differentiation.

Figure 7 below shows images of typical mESC (mouse embryonic stem cells) colonies in semi-solid culture.

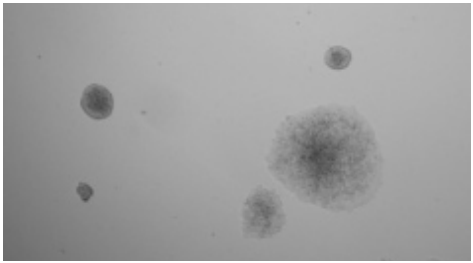


Figure 7: Colonies of CGR8 mESC grown in semi-solid media prepared from *CloneMatrix* and imaged on day 5 post-plating (100x, bright field).

The correct density of colonies in the culture dish is key to the success of colony selection and the automated picking process (25-50 colonies / ml of semi-solid media are recommended as optimal). It is therefore crucial to thoroughly **optimise the seeding densities**.

Seeding densities required to achieve an optimal density of colonies depend on the inherent plating efficiency of the cells used as well as the viability and growth phase of the cell culture at the time of plating.

Evaluate a range of seeding densities in the region of around 500 cells/ml during your optimisation.

Prepare semi-solid media from *CloneMatrix* and a suitable 2x concentrated liquid media as described in section 1 above and supplement with serum (e.g. IMDM, 20% FCS). Add an appropriate amount of LIF (leukemia inhibitory factor) to control the level of cell differentiation (e.g. 1-2µl of $1x10^6$U/ml LIF per 1ml of semi-solid media).

Based on the density and viability of the cell suspension, add the correct amount of cells (ideally in less than 100µl culture volume) to the semi-solid media. Invert the tube several times to gently distribute the cells evenly throughout the semi-solid media.

To plate out;
Dispense ca. 2ml per well of a Genetix PetriWell-6 Plate and 9ml of media for every Genetix PetriWell-1 Plate or 100mm tissue culture dish. This may be achieved using a 10ml pipette or by pouring.

Tilt the culture dish gently to ensure even distribution.

Place in an incubator at 37°C, 5% CO_2 for 3-7 days to allow colonies to grow.

Leave undisturbed for at least 3 days.

Verify the presence of growing colonies at a suitable time point using a light microscope.