Mouse Embryonic Stem Cells

Contents:

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Introduction

ES cells are derived from the inner cell mass (ICM) of a three and a half day old mouse embryo referred to as a blastocyst. Pluripotency is that special quality of ES cells that gives them the potential to develop into all cell types or produce a whole animal. The creation of a mouse model via the genetic engineering of ES cells is currently the tool of choice for determining the function of your sequence of interest in the context of a whole organism, its role in disease, and the generation and testing of therapeutics. Mouse ES cells are amenable to one of the most powerful methods of genetic engineering: gene targeting. This method allows the researcher to make precise modifications to their sequence of interest and then generate a mature adult mouse carrying the engineered alteration. The utility of this technique reaches far beyond the familiar knockout mouse. Its applications include the creation of mutations that mimic human disease, probing for the active site(s) of proteins, investigation of protein/protein interactions, determining the role of regulatory sequences, and much more.

Although not covered in this manual, ES cells are also useful in cell culture studies. Their ability to maintain a normal karyotype for innumerable cell divisions, as well as their untransformed status makes them especially appealing. Protocols have been created that allow for the *in vitro* differentiation of ES cells into many cell types including neuronal, cardiac and epithelial.

The purpose of this manual is to provide a quick reference and general guide for the care and maintanance of ES cells, as well as their employment in gene targeting. Several references are provided at the end for more detailed information.

Protocols for the care of ES cells

Notes: It is extremely important to keep ES cells frozen at –140°C (typically under liquid nitrogen) until needed. The concept we use for tissue culture cells is to ‘freeze slowly – thaw quickly’. It is crucial to use aseptic technique when handling ES cells.

General Concerns

1. It is strongly recommended that ES cultures be monitored daily. ES cells require far more attention than most other tissue culture cells. They are unlikely to be recovered once allowed to differentiate.

2. Those actions that will likely cause ES cells to differentiate include but are not limited to:
   a. Plating too sparsely (<0.5x10^5 cells per cm^2).
   b. Plating too densely (>4x10^5 cells per cm^2).
   c. Failure to change the media when needed. Strive to keep the pH indicator red to red-orange at all times, never yellow.
   d. Allowing the cells get too confluent.
   e. Allowing the culture to consist of a few large colonies rather than several smaller colonies.
   f. Allowing the colonies to get too large.
   g. Failing to add the appropriate amount of LIF (Leukimia Inhibitory Factor) to the culture media.

3. ES cells maintain their pluripotent state best when seeded as a single cell suspension at a rate of approximately 1.5x10^5 - 4x10^5 cells per cm^2.

4. It is likely you will have to split your cells every two to four days.

5. Generally ES cells are split no less than 1:4 and no more than 1:10.

Please refer to the Photo Gallery for visual guidelines of cell density and differentiation.

Thawing ES cells

1. Using the predetermined surface area that the cryotube of ES cells will cover (see surface area table), decide which size plate you will be seeding the cells onto. Label the plates accordingly.

Notes: the area should already be coated with feeder cells.

2. Place 5ml of ES cell media into a 15ml conical tube.

3. Remove the cryotube from the freezer and place it IMMEDIATELY into the 37°C water bath making sure not to submerge the cryotube above the base of the cap.

4. Carefully monitor the media in the cryotube until there is only a small crystal of ice present. At this time take the cryotube into the hood.

5. Using a 1ml pipette, carefully pipette the media up and down two to three times ensuring that any cells settled to the bottom of the cryotube are resuspended (the ice crystal will have melted by now).

6. Place the ES cells into the 5ml of ES cell media you have placed in the 15ml conical tube and mix gently.

7. Pellet the cells by centrifugation at approximately 340xg for 3 minutes. Be careful not to spin the cells too forcefully as this will damage them.

8. Carefully aspirate the supernatant.

9. Resuspend the pellet in the appropriate amount of media and aliquot to the predetermined surface area.

Feeding ES cells

1.ES cells will generally require feeding every day.

2.Carefully aspirate the old media from the plate by placing your pipette along the side of the plate, well, or flask while tilting.

3.If you detect a large amount of dead cells or debris on the plate (as is commonly the case the day after thawing) you can gently rinse the cells with PBS and aspirate. **Caution:** ES cells can easily be washed off of the feeder layer.

4.Apply fresh ES cell media to the plate by placing your pipette along the side of the plate, well or flask and gently dispensing the media.

Splitting ES cells

1. ES cells generally should not be split at ratios lower than 1:4 or higher than 1:10. Cells should be split when they reach ~80% confluency. Please see the photographs in the photo gallery taken at 48 hours, of ES cells plated at $4x10^5$ cells per cm^2 for an approximation of their appearance.

2. Aspirate the media from the tissue culture dish.

3. Gently rinse the dish with PBS to remove any residual serum (the presence of serum will inactivate trypsin).

4. Add enough trypsin (0.25% Trypsin in 0.1% EDTA) to just cover the surface area (for example: 1ml of trypsin will cover a 100mm diameter dish).

5. Place the dish in the 37ºC, 5% CO_2, humidified incubator for 3-5 minutes or until cells release from the bottom of the dish (this should be visible with the naked eye). Beware that extended exposure of ES cells to trypsin is toxic. Keep the time to a minumum.

6. Remove the dish from the incubator and observe the cells under the microscope. Ensure that colonies have released from the plate and have separated into single cells. You may have to apply some gentle agitation in order to break up the colonies.

7. Continue with the trypsinization until approximately 70% of the cells are single cells.

8. In the hood, add 6-10 times the volume of ES cell media as trypsin (for example: if you added 1ml of trypsin you now need to add 6-10ml of ES cell media).

9. Pipette the cells up and down a few times while rinsing the dish in order to ensure that all the culture is in a single cell suspension.

10. Collect the cells into a clean tube.

11. Centrifuge at no more than 340xg for approximately 3 minutes to pellet the cells.

12. Resuspend the cells in the desired volume of ES cell media and aliquot to new dishes. Make sure the cells are evenly dispersed across the surface area of the dish. Do this by gently moving the dish back and forth and side to side, never swirl in a circular manner, as this will cause the cells to accumulate around the perimeter of the dish.

Freezing ES cells

1. ES cells are generally frozen down and thawed from and to the same surface area provided the area was confluent at the time of freezing. For example: an 80% confluent 100mm dish (having $55cm^2$ of surface area) can be frozen down into 1ml and thawed back to a total surface area of $55cm^2$.

2. ES cells can be frozen at any time, although generally cells are frozen down when they would normally need to be split (~80% confluency).

3. To freeze ES cells; first typsinize the cells into a single cell suspension. Pellet the cells by centrifugation as above. Freeze the cells down in a 4:6 ratio of ES cell media to ES cell freezing media. For example: first resuspend the ES cell pellet in 0.4ml ES cell media. Add 0.6ml ES cell freezing media for a final volume of 1ml. Mix, and place 1ml into a labeled cryotube.

4. Place the cryotubes containing the cells to be frozen into a storage box, place the box into a cooler, place the cooler in a –80ºC freezer and leave them overnight. Transfer the cryotubes to cryostorage at -140ºC (typically under liquid nitrogen). This process allows the cells to undergo a slow decline in temperature until frozen.

Common Tissue culture surface areas

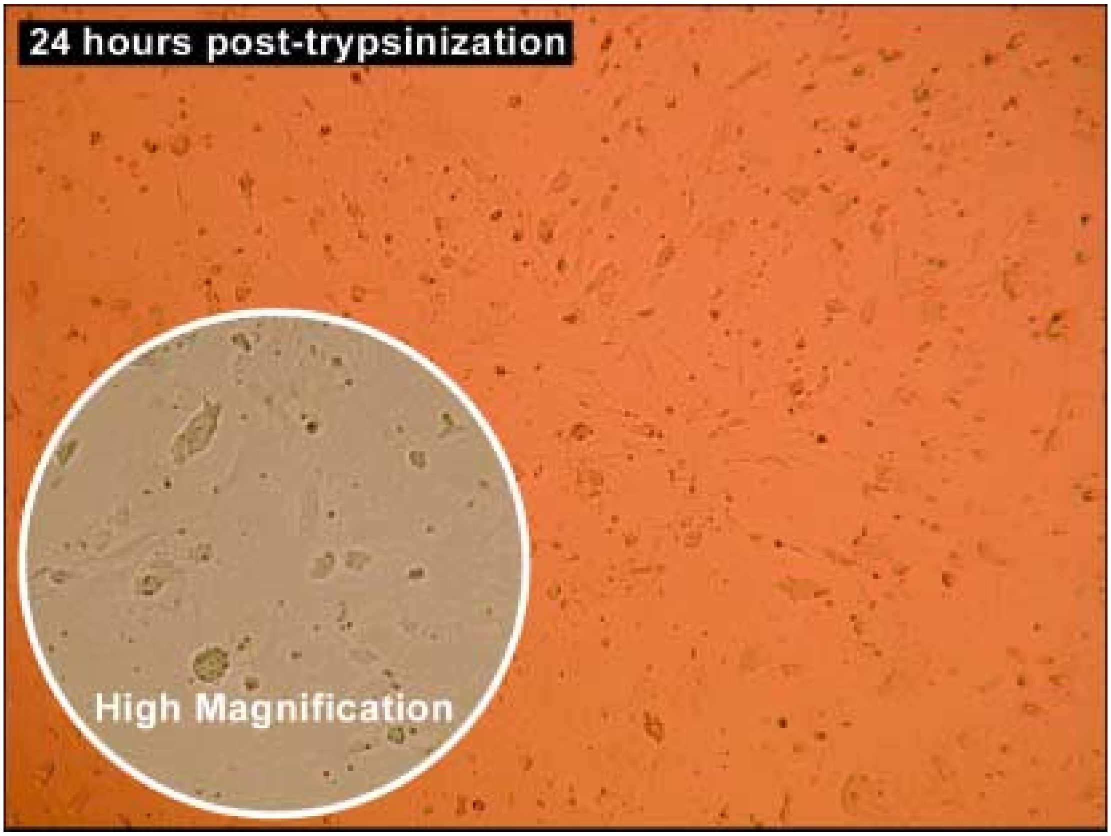
Corning or Costar Flask size	Growth Area (cm^2)
T25	25
T75	75
T150	150
T162	162
T175	175
T225	225

Treated Tissue Culture Dish Size diameter (mm)	Growth Area (cm^2)
35mm	8
60mm	21
100mm	55
150mm	148
245mm	500

Plate Size	Growth Area (cm^2) for single well	Growth Area (cm^2) for entire plate
6 well	9.5	57
12 well	3.8	45.6
24 well	1.9	45.6
48 well	0.95	45.6

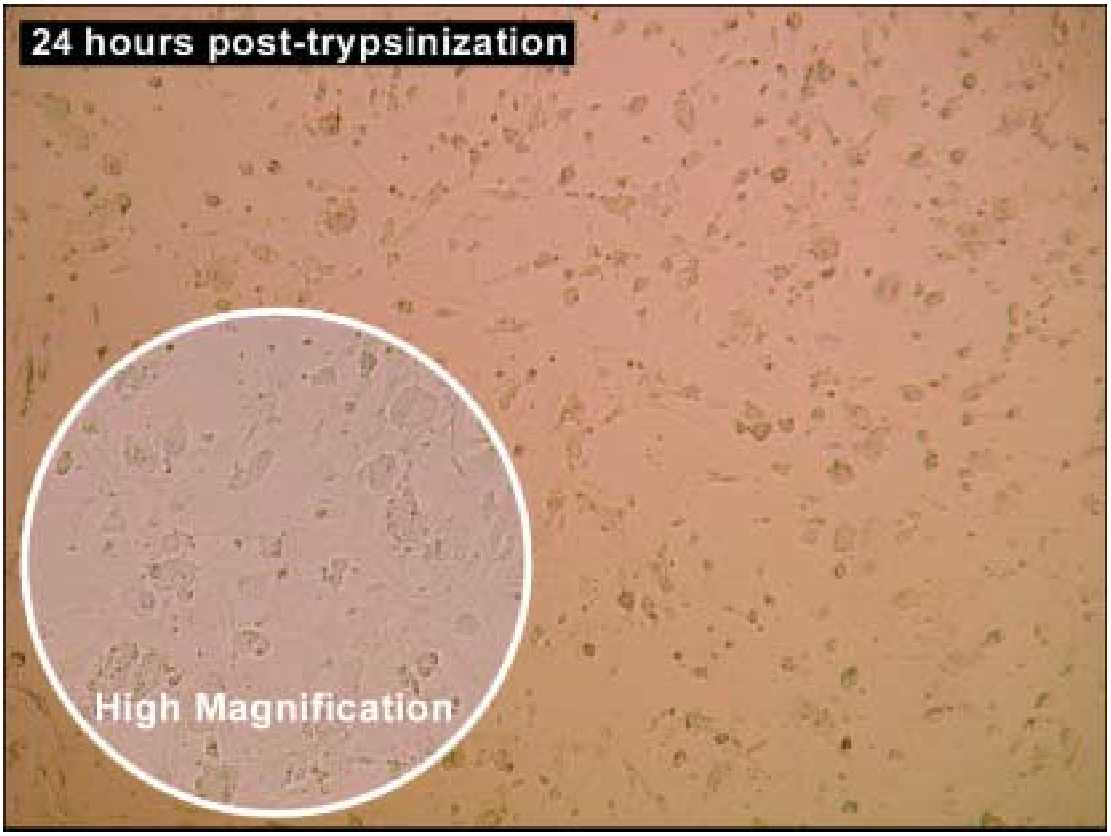
Photo Gallery

ES cells seeded too sparsely at 0.5 x 10^5 cells per cm^2

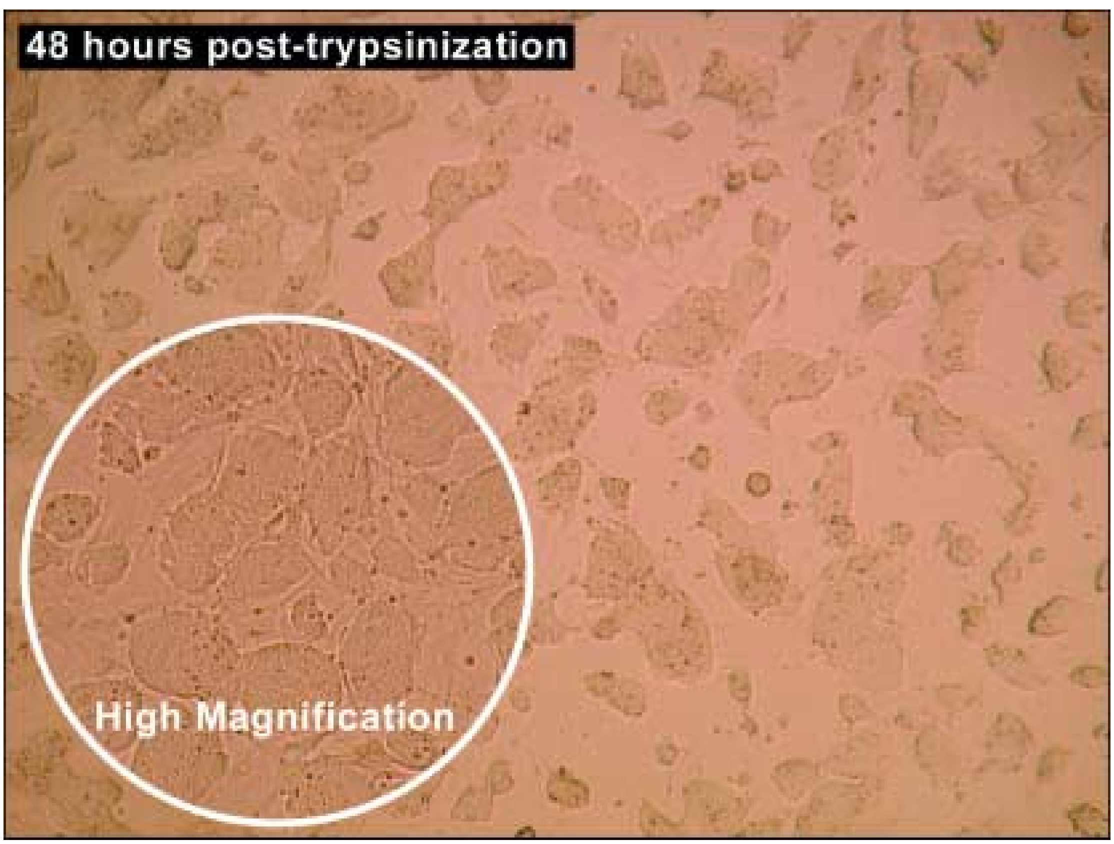


ES cells seeded too sparsely at 0.5 x 10^5 cells per cm^2

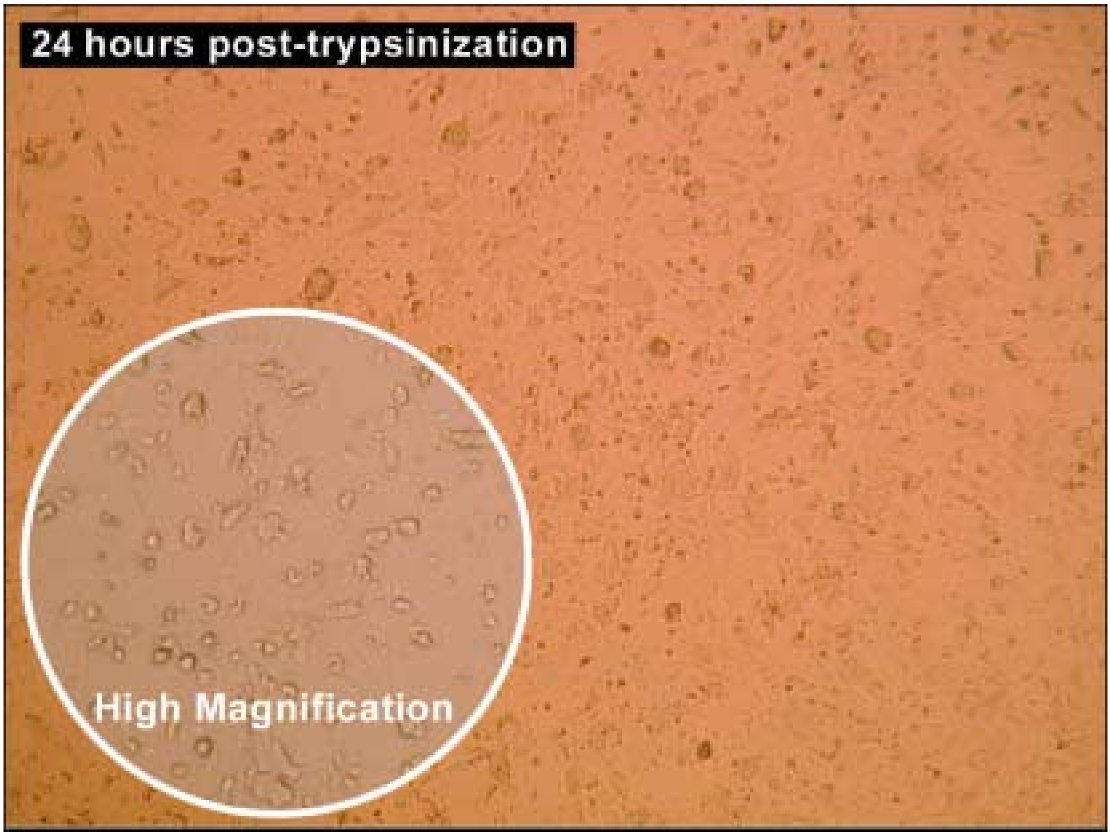
ES cells seeded at 1.5 x 10^5 cells per cm^2



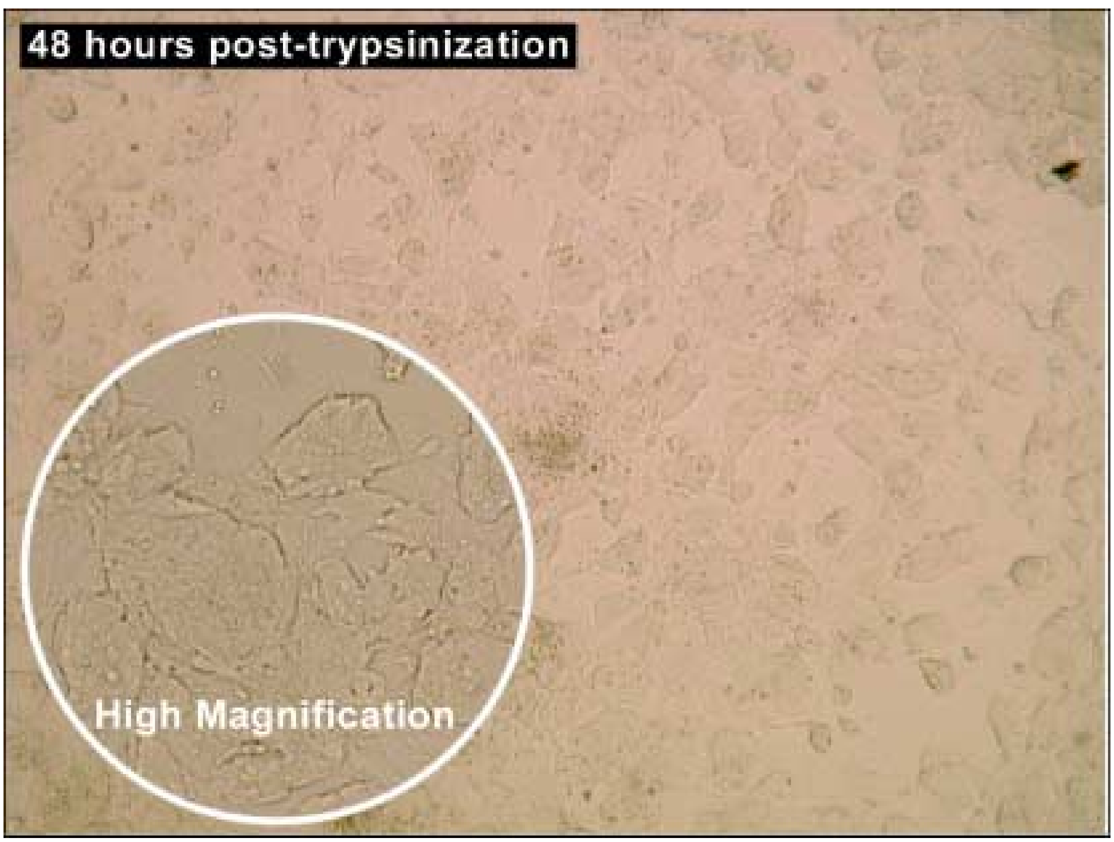
ES cells seeded at 1.5 x 10^5 cells per cm^2



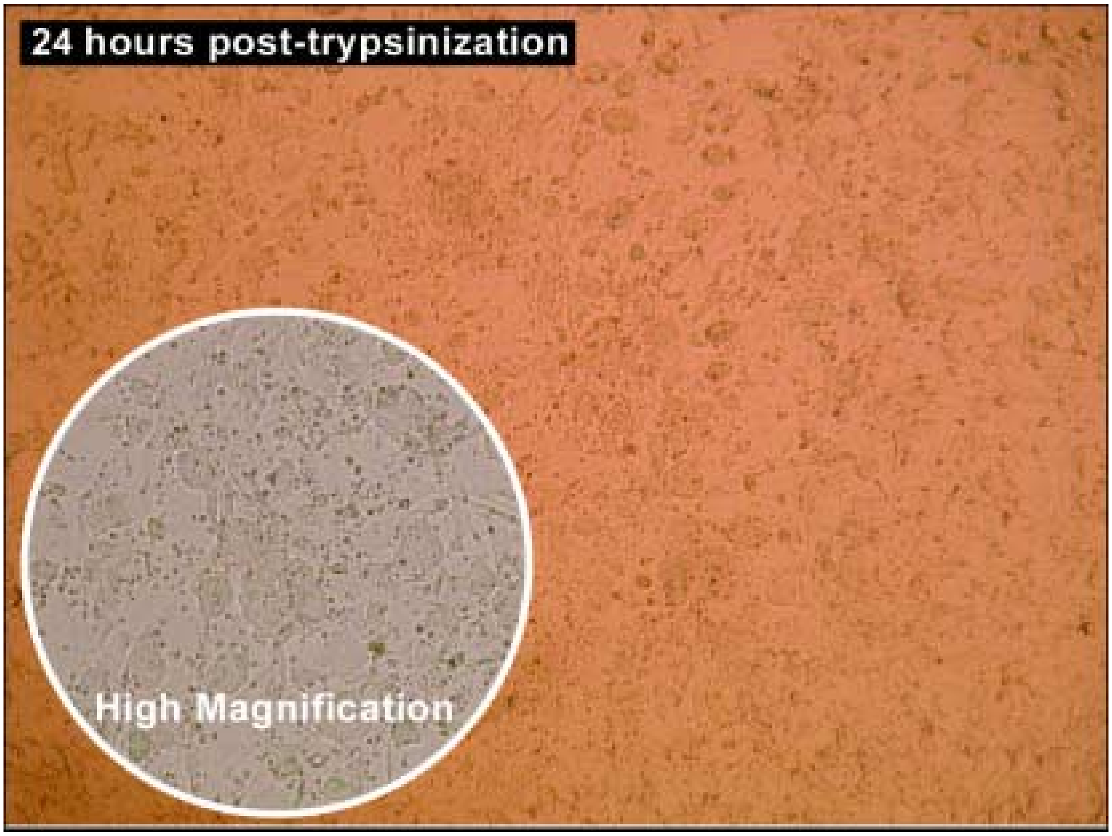
ES cells seeded at 2.5 x 10^5 cells per cm^2



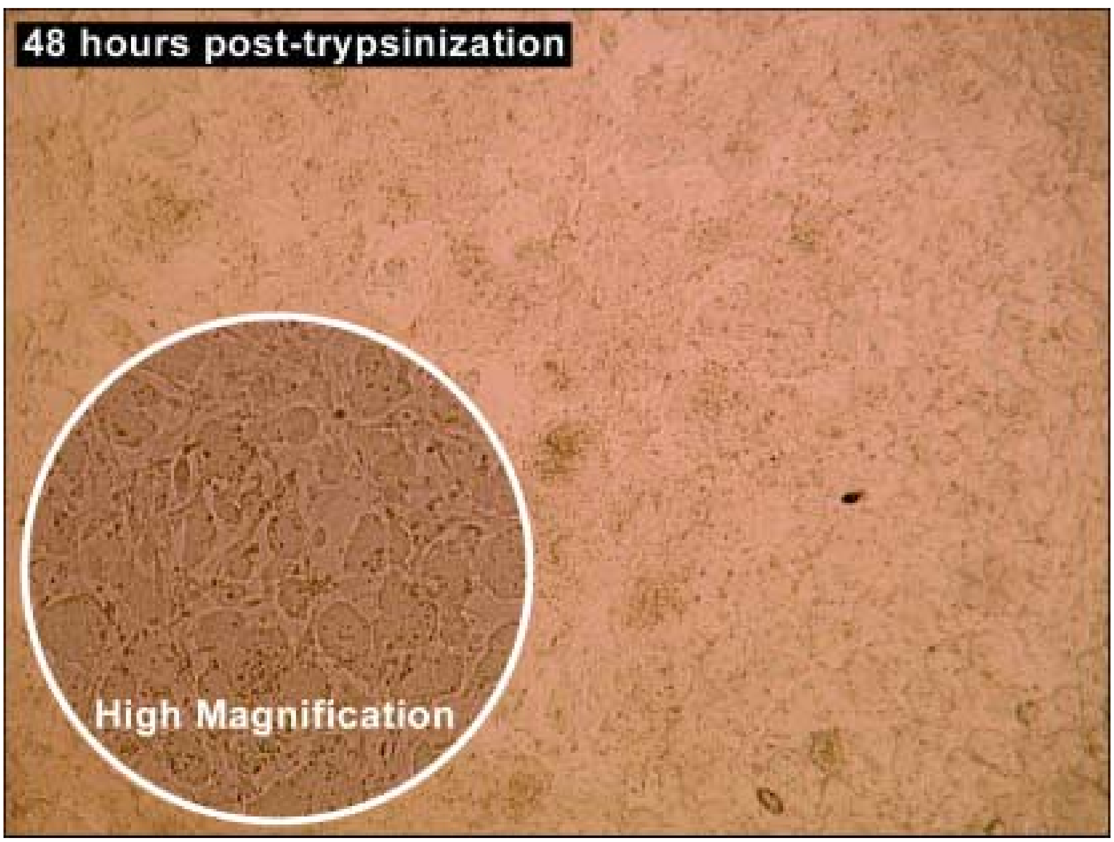
ES cells seeded at 2.5 x 10^5 cells per cm^2

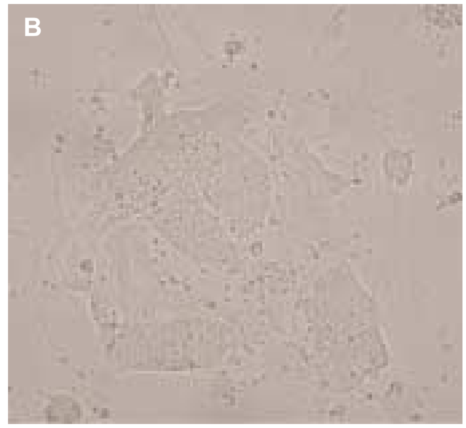


ES cells seeded at $4x10^5$ cells per cm^2



ES cells seeded at $4x10^5$ cells per cm^2





A. Differentiated ES cells are shown within the circle.

B. Notice the extreme flattening, irregular shaping, and mottled appearance of the cells in this differentiated colony.

C. Typical morphology of embryonic mouse fibroblasts (feeder cells).

Media Recipes

	ES Media			***ES Freeze Media***		
Component	1 liter	500ml	250ml	500ml	250ml	100ml
DMEM	800ml	400ml	200ml			
FCS	150ml	75ml	37.5ml	410ml	205ml	82ml
L-Glu	20ml	10ml	5ml			
NEAA	10	5	2.5			
NaPyr	10	5	2.5			
P/S	10	5	2.5			
β***-me***	7µlΩ	3.5µlΩ	1.75µlΩ			
LIF	‡	‡	‡			
DMSO				90ml*	45ml*	18ml*

Note: FCS needs to be heat inactivated for ES freeze media. Heat inactivation is accomplished by incubating FCS at 56ºC for 30 minutes followed by a gradual cooling to room temperature.

* When making ES freeze media, always place the heat inactivated FCS into the bottletop filter prior to the addition of DMSO.

‡ LIF should always be added to ES cell media. If using commercial LIF the amount to use should be indicated with the product. If using non-commercial LIF the amount will have to be determined empirically by titration.

Ω Never add β-mercaptoethanol directly to a bottletop filter. Only add it in conjunction with other fluids.

Abbreviations: DMEM - Dulbecco's modification of eagle's medium, FCS - fetal calf serum, L-Glu - L-glutamine, NEAA - non-essential amino acids, NaPyr - sodium pyruvate, P/S - penicillin streptomycin, β-me - βeta mercaptoethanol, LIF - leukemia inhibitory factor, DMSO - dimethyl sulfoxide

Media components can be purchased from the following companies as well as several other suppliers:

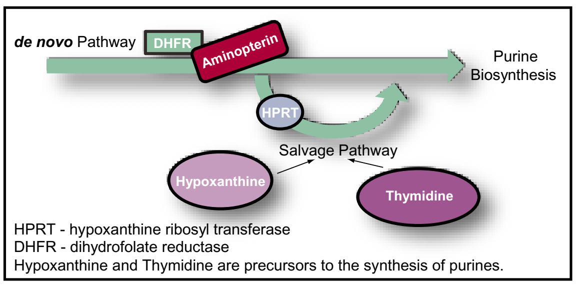
Component	Company	Catalog Number
DMEM	Fisher	10-013-CM
L-glutamine	VWR	45000-676
NaPyr	Fisher	25-000-CI
NEAA	Fisher	13-114-E
P/S	Fisher	30-001-CI
β-me	Sigma-Aldrich	63690
DMSO	Calbiochem	317275
FCS	Hyclone	SH30396.03

Drug Selections

HAT (Hypoxanthine, Aminopterin, Thymidine) selection

What is HAT used for?
HAT is a positive selection FOR the presence of the HPRT protein. The *Hprt* gene is found endogenously in mammalian cells. This makes it somewhat unique among drug selectable markers. In light of this fact, to use *Hprt* as a positive marker in a targeting construct you must start with *Hprt* negative ES cells. HAT selection may be initiated 24 hours after electroporation. HAT selection is rapid, as compared to other drug selections, killing the vast majority of *Hprt* positive cells in the culture in less than 3 days. HAT resistant colonies should become visible to the naked eye by approximately day 10-12 of selection. Colonies are generally picked after 12-14 days of selection.

Mechanism of Action:



There are two metabolic pathways for the synthesis of purines; *de novo* and salvage. Aminopterin (A in HAT) is the active component of the system. Aminopterin blocks the activity of DHFR, a critical enzyme in the *de novo* pathway. When this happens the cell will be forced to use the salvage pathway for purine biosynthesis. If HPRT is present, along with the necessary precursors, hypoxanthine, and thymidine, the cell can synthesize purines and survive. If HPRT is absent, the salvage pathway is not functional and the cell dies, being unable to synthesize purines.

Important Note: ES cells cannot be abruptly taken off of HAT media. To remove ES cells from HAT selection simply provide the cells with HT supplemented media for a period of approximately 5 days. HT supplement can be purchased as a powder stock from Sigma-Aldrich. This is done in order to allow time for the residual aminopterin to be cleared and for the cells to produce enough DHFR and other enzymes for the *de novo* pathway to function efficiently. If hypoxanthine and thymidine are not provided during this interim time the cells will die due to their inability to synthesize purines at the levels required.

6-thioguanine selection

What is 6-thioguanine used for?
6-thioguanine is used as a negative selection AGAINST the presence of HPRT. This type of selection can be used in the targeted disruption of the endogenous *Hprt* locus. It is also frequently used as a component of the 'tag-and-exchange' gene targeting strategy, as well as others.

Mechanism of action:
A functional HPRT protein will take up 6-thoiguanine and metabolize it into its toxic form. This newly formed toxic nucleotide will ultimately be incorporated into the DNA of the cell leading to its death. Those cells without functional HPRT will not be harmed since they are unable to metabolize the 6-thioguanine into its toxic form.

Important note #1: *Hprt* positive ES cells that are under 6-thioguanine selection will release the toxic nucleotides they have formed into the media. They may also cross-feed their toxic nucleotides to neighboring cells. Therefore, when performing a 6-thioguanine selection be sure to have the cells freshly seeded into 6-thioguanine containing media as a somewhat dilute, single cell suspension. Also be careful to change the media daily during the first few days of selection. Also, be sure the feeder cells being used are *Hprt* negative. All this will increase the chances that only *Hprt* positive ES cells die and the desired *Hprt* negative ES cells survive.

Important Note#2: When selecting for the loss of *Hprt* as part of a targeting strategy, it is important to delay exposure to 6-thioguanine until enough time has passed for residual HPRT protein to be degraded. This needs to be empirically determined, as the integration site of the *Hprt* gene will in part determine its expression level and hence the amount of protein still in the cell at the time the gene is lost. Generally speaking, 3-6 days is an appropriate range to begin selection. Colonies are usually picked after 12-14 days of selection.

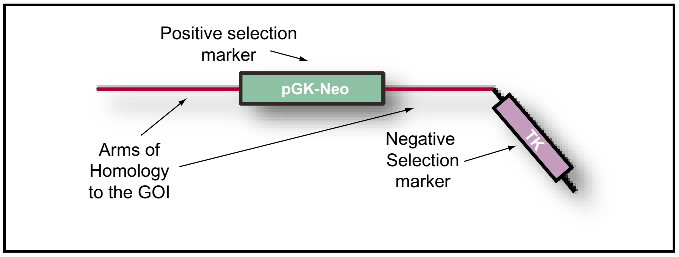
Positive-negative selection using neomycin and thymidine kinase (TK)

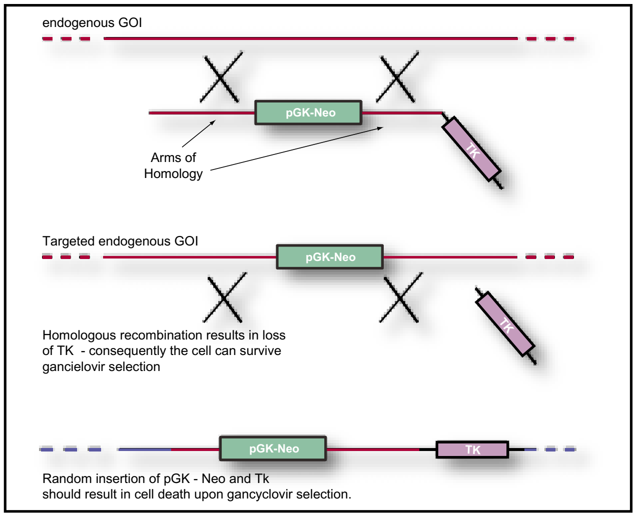
What are G418 and ganciclovir used for?
The neomycin gene confers resistance to G418 while TK renders cells susceptible to ganciclovir. Neomycin is used as a positive selection marker while TK is used as a negative selection marker. Generally, they are used together in a strategy commonly referred to as 'positive-negative selection'.

Mechanism of action:
G418 binds to the 30S or 50S subunit of the ribosome causing miscoding. During protein synthesis it inhibits initiation and elongation. Ganciclovir is a pro-drug nucleoside analog that is activated by phosphorylation. When a viral thymidine kinase suicide gene is expressed, it will convert the non-toxic pro-drug to its phosphorylated toxic analog. This toxic analog is then incorporated into the DNA of dividing eukaryotic cells bringing about their death.

Important note #1: As the mechanism of ganciclovir is reliant on replication, actively multiplying cells will be affected sooner than those that are not actively dividing.

Important note #2: Neomycin positive ES cells have variable resistance to G418. Therefore it is recommended that varying concentrations of G418 be tested. For mammalian cells a concentration of 400ug/ml can be used as a guide. It may take to 3-7 days for the selection to become apparent. ES cells should be placed in media containing G418 and ganciclovir 24hrs after electroporation.





Hygromycin selection
What is hygromycin used for?
Hygromycin is used as a positive selection marker in gene targeting.

Mechanism of action:
Hygromycin blocks peptide synthesis and inhibits elongation.

Puromycin selection
What is puromycin used for?
Puromycin is used as a positive selection marker in gene targeting

Mechanism of action:
Puromycin is a nucleoside antibiotic. It is a protein synthesis inhibitor that causes premature chain termination by acting as an analog of the 3' terminal end of aminoacyl-tRNA. Puromycin prevents growth of ES cells and acts very quickly, with the potential to kill 99% of cells in just 2 days. The resistance gene puromycin acetyltransferase (*pac*) gives very effective protection.

Drug selection table:

Selecting for	Drug used for selection
Hygromycin	Hygromycin
Puromycin	Puromycin
Neomycin	G418
Hprt negative	6 -thioguanine
Hprt positive	HAT
TK negative	Ganciclovir

Note: all drugs listed can be purchased from Sigma - Aldrich

Some notes on strain background
After 20 generations of brother sister matings, on average at least 98.6% of the loci in each mouse are homozygous. The following information concerning strain background is taken from: J A Beck, S Lloyd, M Hafezparast, M Lennon-Pierce, J T Eppig, M F W Festing & E M C Fisher *Geneologies of Mouse Strains*, Nature Genetics **24**, 23–25 (2000) (an excellent reference).

“Many strains have been inbred for more than 150 generations and are essentially homozygous at all loci. Each inbred strain is also isogenic (genetically identical) because all individuals trace back to a common ancestor in the twentieth or a subsequent generation. Many inbred strains are bred for specific phenotypes. For example, senescence-accelerated mice (SAM) display characteristics of an increased rate of ageing. C57BL/6 has an increased preference for alcohol and narcotics and is used in studies of the genetics of substance preference. Some inbred strains have features that are advantageous to transgenic and embryonic stem (ES) cell technology: the large pronuclei of FVB mice are useful for gene transfer experiments involving direct DNA injection of the fertilized egg, and 129 ES cells are particularly successful in germline transmission. The sensitivity of BALB/c and C3H mice to mutagenesis by ethyl nitrosourea (ENU) has been valuable in mutagenesis programmes (Mouse mutagenesis consortium, http://www.mgu.har.mrc.ac.uk/mutabase/; German Human Genome Project, (http://www.gsf.de/isg/groups/enu-mouse.html). Inbred strains with distinctive behavioural characteristics are widely used in neuroscience. Phenotypic differences between inbred strains need to be taken into account when designing experiments. Strains may vary in characteristics that may not be relevant to the phenotype being studied, but which may indirectly influence experimental results.”

Gene targeting

Initial considerations when preparing a targeting construct

1. Length of homologous arms. The following graph illustrating the relationship between the length of homology and gene targeting frequency is taken from P Hasty, J., Rivera-Perez, *et.al*., *Mol. Cell. Biol.* **11**, 5586-5591 (1991):

2. Linearization of the targeting construct is important for obtaining homologous recombinants. Linearize the DNA at a site in the backbone of the plasmid, not within the homologous region or area of interest. Reserve a single cutting site for this purpose in your strategy. Perform a large scale, overnight digestion for linearization.

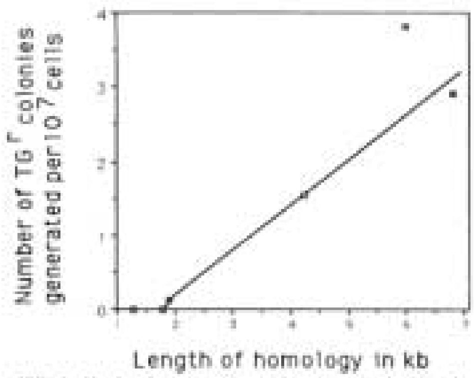


FIG. 1. Absolute frequency of recombination plotted against the length of homology to the target sequence with replacement vectors.

3. The DNA used should be clean and of high purity. Use a method of plasmid DNA isolation that is high quality, such as CsCl banding. Linearized DNA can be prepared for electro poration simply by ethanol precipitation, drying in the hood, and resuspension in sterile TE.

Notes: never turn the UV germicidal light on while DNA is in the hood.

4. Use a final concentration of plasmid no less than 3nM for each electroporation event.

5.Ensure that all downstream screening strategies are in place (i.e. probes for Southern blotting, restriction enzyme sites, PCR primer sites). See more details below under Screening for Targets.

Growing ES cells for electroporation
Electroporation requires a large number of cells. The normal yield of an 80% confluent 10cm dish (55cm^2) of ES cells is approximately $3x10^7$. Feeding the ES cells to be electroporated approximately 4 hours prior to electroporation will increase the likelihood of obtaining homologous recombinants. This is because homologous recombination occurs at a higher rate in cells that are actively undergoing mitosis.

Electroporation of ES cells
1. Follow the manufacturers protocol for the electroporation of ES cells.

2. Place the dishes in the 37ºC, 5% CO^2, humidified incubator.

Screening for targets

There are basically three types of integration events that can occur when DNA is electroporated into ES cells: random integration, insertion or single crossover, and double crossover. It is important that your screening strategy be able to distinguish between the types.

Non-homologous recombination occurs at a far higher frequency than homologous recombination. Random integration is synonomous with non-homologous recombination and therefore represents the single biggest obstacle to finding homologous recombinants. Many strategies for minimizing background due to random integration are commonly used, neo/TK for example. In spite of these efforts, some level of background is inevitable. Screening out random integrants that have survived drug selection is usually done by positioning a probe for Southern blotting outside the region of homology and having a corresponding restriction site that lies beyond the probe. Using only restriction sites within the homology arms of the targeting construct will not differentiate between homologous and non-homologous recombinants. Also, using a probe within the targeting constructs homology arms will give many random bands on a Southern blot making the readout difficult.

Sometimes, it is possible to engineer PCR primers that will give a unique product only when homologous recombination has occured. This approach can save a great deal of time but should not be the sole source of validation. It is important to check both flanks for correct junctions as single crossover events can occur yielding results that appear correct when one flank is tested but are not correct on the remaining flank. Double crossover events can only be confirmed by testing the junctions between both flanks of the targeting construct and the genomic sequence.

Tetraploid blastocyst injection vs. Diploid blastocyst injection

There are currently two methods of generating gene targeted mice; diploid and tetraploid blastocyst injection. Diploid blastocyst injection has been widely practiced for many years while tetraploid blastocyst injection is relatively new. Consequently, diploid blastocyst injection currently has a higher success rate per blastocyst injected but as time goes on tetraploid blastocyst injection is becoming more and more efficient. Obtaining a multitude of genetically identical animals is possible with either technique. With diploid blastocyst injection genetically identical animals are obtained by mating a high percentage germline transmitting chimera. The resulting F1 animals are all genetically identical. Tetraploid blastocyst injection gives genetically identical animals directly from the foster mother saving a generation of mating to accomplish this goal.

However, this technique generally requires the use of F1 hybrid ES cell lines and a great deal more injections and foster mothers than the more traditional diploid blastocyst injections.Therefore, the choice of technique greatly depends on the desired outcome as well as the available personnel. For the novice, diploid blastocyst injection is likely the best choice.

Feeders vs. gelatin

Feeders are used in the culturing of mouse ES cells. They provide both a substrate for the ES cells to grow on and they secrete many factors necessary for ES cells to maintain pluripotency. Sometimes people will use gelatin to coat their growth surfaces in the place of feeders. While this certainly works for some applications it is not advisable for those wanting to use the cells for blastocyst injection and mouse production in the future. It is widely considered a necessity to grow ES cells for future mouse production on feeders. It is thought that feeders provide for ES cells in ways that gelatin alone cannot. It should be noted that many labs use both gelatin and feeders in combination. This strategy provides the maximum amount of substrate for the ES cells and may very well be the best option.

Leukemia inhibitory factor (LIF)

LIF is one factor produced by feeder cells that has been experimentally shown to help maintain ES cells in their pluripotent state. It is essential to maintaining healthy pluripotent ES cells. LIF is generally provided in part by the feeder cell layer but also needs to be included as a media supplement.

Using BACs for gene targeting

In the past, the size of a gene targeting vector has been limited to that of a plasmid, since this was the only form which was readily amenable to molecular cloning techniques. However, today, the modification of BAC clones using ET-cloning or ‘recombineering’ as it is commonly called, is proving to be a powerful tool for the generation of gene targeting constructs.

Precise changes in sequence can be made accurately, efficiently, and with tremendous ease using this technology. In addition to the cloning utility of this technique there are advantages to using a BAC in the gene targeting reaction itself. Due to the large size of a BAC, and hence the extended length of the homologous arms, the need for isogenic DNA is virtually abolished. This makes it possible to easily target ES cell lines from multiple strain backgrounds with a single construct. See the references regarding BACs and gene targeting below for more information.

Conclusion

In summary, while ES cell culture and gene targeting require stringent adherence to certain ground rules; given diligent and dedicated care ES cells can certainly result in the successful generation of genetically altered mice.

ES cell lines available from Open Biosystems:

Cell Line	Strain	Tetraploid or Diploid Use
EL M3	129 x 129	Diploid
v6.5	C57BL/6(F) x 129/sv(M)	Tetraploid or Diploid
v26.2	C57BL x C57BL	Diploid
v17.2	BALB/c (F) x 129/sv(M)	Tetraploid or Diploid
v39.7	BALB/c (F) x BALB/c	Diploid

References

BACs and gene targeting

Cotta-de-Almeida, V., Schonhodd, S., Shibata, T., Leiter, A. and Snapper, S.B. A new method for rapidly generating gene-targeting vectors by engineering BACs through homologous recombination in bacteria *Genome Research* **13**: 2190-2194 (2003)

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