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Overview of ES Core Facility

Our Mission

The ES Core Facility (ECF) was founded by the NINDS Core Center Grant and was established to benefit the contributors of this proposal. The mission of ECF is to effectively produce ES cell lines with a high probability of germline transmission.

Core Service

Services provided by the Core for a typical project include:

- Provide guidance on the design of targeting construct
- Generate targeted ES cell lines for the production of chimeric mice
- Karyotyping ES cells to be micro-injected into blastocysts

Consultation is available from ECF directors and staff members on the entire procedures of generating gene knock-out mice.

Application for Service

Prior to the initiation of a project, a brief meeting is generally required between the investigator and ECF facility staff resulting in a mutually acceptable research strategy. This strategy will outline specifics of the project including knockout strategy, KO construct design, screening assays, and other procedural issues relevant to the generation of targeted ES cells. In addition, a completed service application form, signed by the principal investigator and approved by the Core Director, will also be required. The Core Director will prioritize the service requests according to the difficulty of the project and work load.

Contact Information

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Generation of Targeted ES Cell Lines

Our ES facility will generate targeted ES cell lines with KO constructs provided by investigators. The task is subdivided into 3 phases:

Phase I: Electroporation (approx. 2-3 weeks)

ES cells will be electroporated using the investigator's construct. The cells will then be plated and selected using a drug appropriate to the marker used in the construct. After ten days of selection up to approximately 200 colonies that appear normal will be picked and spilt onto 2 96 well plates. One plate, the master plate, will be frozen to be used to expand positive clones. The second will be used for the screening of the clones for the gene target. The investigator will be given 96 well plates containing DNA isolated from ES cells suitable for screening using a mini-Southern or PCR.

Notes: The Core cannot guarantee how many clones will be picked or will expand properly. For most constructs the standard procedure of picking up to 200 clones per electroporation will be sufficient.

Phase II: Expansion of Positive Clones (approx. 1-2 weeks)

After screening the clones in Phase 2, all positive by PCR clones will be expanded on 24 well plates (up to 24 clones). The investigator will then receive a cell pellet from each clone sufficient to use for Southern analysis for further characterization and one vial of each clone will be frozen for LN2 storage.

Notes: The Core cannot guarantee that any of the clones will be properly targeted. Colonies picked appeared undifferentiated, and as they survived drug selection, are assumed to contain the construct in some form. The lengths of the arms and the sequence fidelity (and some luck) have the greatest impact on the number of properly targeted clones produced.

Phase III: Further Expansion & Karyotype Analysis

(approx. 1-2 weeks for karyotype analysis)

Up to 5 of the properly targeted clones will be further expanded initially. They will then be checked for proper chromosome counts (karyotype analysis). The best growing 4 euploid clones will then be expanded further and referred to the Transgenic Core to be placed in the injection queue. If less than 2 euploid lines are identified, a second and, if necessary, third round of electroporation will be performed (so that at up to 500 clones are made available for screening).

For the week of injection, one clone per day will be prepared for injection Tue-Fri. In the case where less than 4 properly targeted euploid clones were made, the best growing clone(s) will be injected over more than one day.

Karyotyping of Positive ES Cell Lines

Cell lines submitted for karyotyping will be examined for the presence of the correct number of chromosomes. For each line 10-20 cell spreads will be examined.

ES Cell Production Request Form

Investigator: _____ Building & Room No: _____

Phone: _____ E-mail: _____

Contact Person: _____ E-mail: _____

Animal Protocol #: _____ Budget #: _____

Construct Name: _____ Drug selection required: _____

Strain of Arms of Homology: _____ Size of arms: _____

Targeted ES Cell Production	Check Service	Services
Electroporation		Electroporation of one 10cm plate of ES cells. Cells will be grown in selective media for 10 days and up to 200 colonies will be picked and grown on 96 well plates. Samples will be sent to the investigator for evaluation, and the colonies will be frozen in the 96 well plate.
DNA isolation		DNA from cells in the sample 96 well plate will be isolated and given to the investigators for evaluation.
Expansion of Positive Colonies		Up to 24 clones selected by the investigator will be expanded into one well of a 24 well plate. One vial of cells will be frozen from each plate, and a sample will be sent to the investigator.
Further Expansion & Karyotype Analysis		Cells chosen for injection will be expanded. 5 vials will be frozen per line. Cell lines will be karyotyped prior to injection.
Karyotyping		Karyotyping of additional cell lines.

Approved by Lab PI: _____ Date: _____

Approved by Core Director: _____ Date: _____

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Principles of Gene Targeting

Gene targeting, the exchange of an endogenous allele for a mutant copy via homologous recombination, is a powerful tool. It allows us to create strains of mice containing known mutations including null and point mutations, conditional mutations, chromosomal rearrangements, deletions of functional domains, exchange of functional domains, and gain of function through insertion of exogenous DNA.

It has been used to create mouse models of disease and to study gene function and expression. As such, targeting has become a standard tool of researchers. This, however, does not mean that the process is easy nor that success is guaranteed. Targeting is a laborious process involving cloning of the gene, construction of appropriate vectors, introduction of the construct into ES cells, selection and identification of targeted cells, injection of targeted cells, and derivation of the resulting mutant mice.

One of the major advantages of targeting a gene using ES cells is the ability to control the location of the mutation. However, homologous recombination occurs about 1000-fold less than random insertion. This is why screening beyond selection for the mere presence of the marker in the target is required. These ES cells once injected into a blastocyst can contribute to development in the embryo creating a chimeric mouse. If the ES cells participate in the creation of germ cells, these mice can be bred to produce strains of “custom” mutant mice.

Transgenes may be inserted into the ES cell genome under the control of the regulatory elements of a particular locus. They may be inserted and leave the endogenous gene functionally intact, or disrupt gene function due to replacement, and may include reporter genes that allow investigators to follow in situ expression.

There is no single strategy that will work for all studies. Which is best for a particular gene is dependent on the problem being analyzed. Before beginning a targeting project, investigators need to plan the strategy for the experiment including the type of mutation desired, the ES cell strain, construction of the targeting vector, and the method of screening.

Investigators utilizing the gene targeting service will be required to produce the targeting construct and screen picked colonies for homologous recombinants using a proven screening assay. The facility will perform the ES cell culture, electroporation, selection, karyotype analysis and expansion of clones for injection.

Requirements for the Design of Targeting Constructs

A basic targeting vector consists of a long arm of homology, a short arm of homology, and a cassette containing the positive selection marker, typically a neomycin resistance gene. There are two general types of constructs that may be used to target genes in ES cells: replacement vectors and insertion vectors.

Replacement vectors contain a part of the genomic sequence targeted (the arms) and a mutant foreign sequence (e.g. a positive selection cassette) within the coding region. Outside of the homologous region, the vector is linearized and recombination occurs as the result of a double crossover event.

Insertion vectors are linear within the region of homology, and are entirely inserted in a single crossover event often resulting in a duplication of the homologous DNA. The location of the linearization site will affect the recombinant allele's structure, and requires consideration of

any RNA splicing that may affect the allele. This can lead to either inactivation or subtle mutations within the gene.

Both designs require homology of the vector and target locus. The frequency of targeting increases with longer homology up to at least 10kb. The arms of homology should be isogenic to the strain of ES cell used, usually 129.

The major parameters that influence the frequency of homologous recombination are the target locus and locus region of DNA, the length of the homologous regions used in the construct, the transcriptional activity of the locus, the permeation of the selectable marker, and the use of isogenic DNA.

Also, you will need to have information about the exons neighboring your insertion points. Exons may fuse together even if the selection cassette is excised out. Ideally for a null mutation, you will want to disrupt the exon that includes the initiation methionine. If this exon is unknown, then insertion of the vector should take place in an exon in the 5' region of the gene.

Orientation of the selection cassette should also be considered. If put in the opposite direction of the original gene, there will be a minimal effect of the exogenous promoter and there will not be strange mRNA produced by your cells.

For the long arm of your vector you will need a minimum of 5 kb and 7-10 kb is preferred. The long arm will contribute most of the homology so, in theory, the longer the long arm the higher the frequency of homologous recombination. However, entire target construct will still need to fit into a vector, and too long a region may contain many enzyme sites, complicating your experiments.

The short arm should be 1-2 kb. Its been reported that a short arm of less than 1 kb decreases recombination, but this will be the part of your vector used for PCR amplification, so you do not want to make it too long. A short arm of over 2 kb may make PCR screening very difficult.

Random insertion can be selected against using a positive-negative strategy. This type of selection usually reduces the number of colonies to be screened 4-10 fold. If positive-negative selection is to be used the negative selection cassette should be located on the end of the short arm.

Regardless of the type of vector constructed, its sequence should be confirmed prior to using it for gene targeting.

Screening Assays for the Identification of Targeted ES Clones

Homologous recombination events need to be identified from the random integration events that will occur during transfection. Vector construction, rapid screening procedures, and advances in cell culture have made it more efficient to identify these clones.

After positive colonies are picked from the selection plates, DNA will be supplied in a 96 well plate format for evaluation of the clones. Typically, this is done using PCR and/or Mini-Southern blotting.

The strategy for using a PCR screen is to amplify a novel junction created only by the homologous recombination event and not by either wild type DNA or random integration. The easiest way to do this is to have one primer that binds to the neo cassette and the other bind to an

endogenous locus just beyond the short arm. Amplification will only take place if the two primers are juxtaposed by homologous recombination having occurred.

In order to test the PCR conditions a control vector will need to be made. This will also serve as a positive control for the ES cell screening. It should include the positive selection cassette in the same direction and site as the targeting vector, followed by a longer short arm that includes the gene-specific primer sequence. The control vector does not need to include the negative selection cassette and the long arm.

This vector may or may not be created at the same time as the target vector, but it should be created early in the process in order to optimize the PCR assay, which may take some time. The primers should be tested using a variety of vector DNA concentrations, and ideally will be sensitive enough to detect 1 fg of DNA. Once satisfied with the sensitivity, the vector should be mixed with genomic DNA and testing again for sensitivity. It would also be a good idea to develop 2 primer sets as one set may work better than the other in the actual ES clone samples.

PCR is an excellent initial screen, but proper targeting should be confirmed by Southern blot prior to injection into blastocysts.

The Southern Blot should be established using several different enzyme digestions using wild type DNA. It is suggested that you design two probes- an inner probe that will identify all recombinants in the ES population, and an outer probe for confirmation of homologous recombination.

The outer probe is gene-specific and is based on the DNA sequences just outside of the targeting vector. Digestion of the DNA should be able to distinguish wild-type versus homologous recombinant based on the molecular weight of the bands. An enzyme that will produce one cut inside the vector and one cut outside the vector is ideal for this.

The inner probe should recognize part of the short arm of the vector. It is also possible to use a probe to the positive selection cassette. This will identify all recombinants in the ES cell lines, including the non-specific ones. If random integration produces a high molecular weight band it may not be detected due to the low amount of DNA transfer. Usually, random insertions can be segregated during the mating process. Use of a negative cassette will help eliminate clones with random integration.

If the same enzyme sites are used for both probes, then it will be possible to probe the blot with the outer probe first, strip the membrane and re-probe with the inner probe.

Overview of ES Cell Culture

ES cells are derived from the inner cell mass of the mouse blastocyst and maintain their ability to contribute to all cell types in the progeny mouse. Precise handling, use of rich media containing LIF, frequent observation of morphology, use of a feeder layer, and frequent passage at low dilution are required to minimize differentiation and the lower the chances of having an abnormal karyotype. The cells should be kept in log phase growth because this is when the cells are most robust and will continue to keep their toti- and pluripotent properties.

Electroporation of ES cells is affected by several parameters that influence viability and efficiency: voltage, ion concentration, DNA concentration and cell concentration. Gene expression and random integration have been correlated to the number of molecules of vector DNA delivered to the cell. Twenty-four hours after electroporation the cells can begin to be selected and 8-12 days later colonies can be picked.

Only ideal colonies should be isolated for screening and future use. The colonies should be slightly smaller than the end of a 20ul pipette tip; edges should be clearly defined with no flattened areas appearing along the edges. Selected colonies are picked into 2 96 well replica plates and continue to be fed with media containing the selective agent. One of the plates will be used to isolate DNA for screening and the other will be used as a master plate. These clones will be frozen in a 96 well plate and stored at -80C while the screening of DNA samples prepared from the clones is performed. These are not ideal long term storage conditions for the ES cells, and so completing the screen in a timely manner is recommended.

Clones that are positive after the initial screening will be thawed and expanded for karyotyping and further evaluation.

If after multiple rounds of electroporation, over 500 colonies are screened and no correctly targeted clones can be identified then it is recommended to re-engineer the targeting vector.

ES Cell Factors Affecting Successful Chimera Production

- **Karyotype**- an abnormal karyotype lowers the probability of germ line transmission
- **Passage Number**- Increasing passage number can increase the likelihood the development of an abnormal karyotype and also decrease the ability of the cells to make chimeras the go germline for other unknown reasons.
- **Cell Morphology/Density**- Cell morphology and confluency are two of the most important factors in chimerism rates. Cells should be dividing at a steady rate and have no signs of differentiation. It is important to have the cells in log phase growth because we are looking for the ES cells to overtake the embryo. When the ES cells are overgrown and have plateaued they do not contribute as large a percentage to the embryo. The result is lower numbers of chimeric animals and lower percentage of chimerism in the individual founders with reduced germline transmission.
- **Mycoplasma Infection**- Mycoplasma can cause chromosome damage.
- **Incomplete Selection**- Contamination of Colonies with wild-type cells decreases the number of targeted cells injected into the embryos.

FAQ

How long will it take?

Under optimal conditions, it may take 11-14 months to obtain homozygous knockout mice. This is based on an estimate of 3-4 months to construct the vector, 1 month to obtain and confirm homologous recombination in the ES cells, 1 month from the injection of the cells into blastocysts until the chimeric mice are obtained, 3-4 months until f1 mice are generated, and 3-4 months until the F1 mating produce homozygotes. Having an efficient screening strategy prior to beginning the ES cell work is essential to quickly identifying positive lines; if this is not worked out before beginning the time to obtain clones will be greatly increased.

What ES cell lines do you have available?

We have both 129 and C57/B16 ES cell lines available. These cell lines have been tested for manipulation (previously targeted) and germline transmission. These cell lines have been also tested for mycoplasma.

How much DNA will I need?

For each round of electroporation approximately 30ug of your targeting vector will be needed at a concentration of 1ug/ul in sterile PBS.

Where can I get more information?

Johns Hopkins ES Core Website: www.?????.org

Johns Hopkins Transgenic Core Website: www.hopkinsmedicine.org/core/Home.htm

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