Module 3: IKMC Resource Overview

Aims
- Understanding knockout design strategies
- Introduction to the IKMC web portal

Introduction
Several large-scale gene targeting and gene trapping projects are participating in the International Knockout Mouse Consortium (IKMC) with the combined goal to mutate each protein coding gene from the C57BL/6 strain and establish a global repository of targeted ES cell lines. Major targeting pipelines include the EUropean COnditional Mouse Mutagenesis program (EUCOMM) and the KnockOut Mouse Project (KOMP). The IKMC also functions as a portal providing information from a variety of resources beyond the ES cells or B6 mouse strain.

In this module you will be introduced to the different knockout strategies used, including traps, deletions and conditional alleles. Navigating the IKMC web portal, the different targeting pipelines will be discussed, identifying individual stages and statuses.
Knockout Resource Overview

The IKMC resource contains a large number of trapped and targeted allele types as shown below:

Detailed descriptions of the various IKMC allele types are available at:

http://www.knockoutmouse.org/about/targeting-strategies

Although some information about conventional alleles, such as gene traps, is provided here, the focus of this workshop will be targeted, conditional resources produced by the KOMP(CSD) and EUCOMM programs. These conditional alleles are arguably the most useful and versatile products of the various pipelines and constitute a majority of the targeted resources.
The first high throughput mutagenesis efforts in the mouse used gene traps, insertional mutations generated randomly in embryonic stem cells. The principle of the method is depicted in the figure below:

A promoterless cassette with a strong splicing acceptor signal, a selectable reporter with stop and polyadenylation signals integrates randomly in the mouse genome. After screening and selection of positive insertions, known sequences in the cassette are used (for example by RACE) to determine the trapped gene.

See a full tutorial at http://www.genetrap.org/tutorials/overview.html

The splicing acceptor in the cassette “traps” the native splicing of the gene causing, ideally, a disruption of the protein product.
Information about all publicly available gene trap cell lines is provided by the International Gene Trap Consortium (IGTC), with links from the IKMC portal. The example below, shows details of more than 20 gene traps available for the Tomm40 gene.

Useful as they are gene traps have a series of disadvantages. First, only genes expressed in Embryonic Stem cells can be disrupted and since the integration of the cassette occurs (more or less) randomly, there is no control as for where exactly in the gene structure the disruption occurs. Methods like recombineering have been developed that allow the high-throughput gene targeting. Together with the completion of the genome sequence and the availability of full genome BAC libraries, the targeting of virtually all protein coding mouse genes becomes a realistic goal.
Knockouts

More recently, large international mouse KO projects have been funded, to provide the scientific community with targeting vector reagents and ES-cells at very low cost-recovery fees. The EU-funded EUCOMM project exclusively uses a conditional strategy to target genes. The NIH-funded KOMP, is divided into two resources, targeting genes using a variety of strategies. The KOMP-CSD consortium (CHORI, WTSI, UC-Davis) targets genes with either conditional KOs or deletions. KOMP-Regeneron targets genes exclusively with deletions. The IKMC was established to coordinate gene targeting efforts and minimise overlap between project gene lists.

The figure below depicts the final goals of each participant by the end of 2011 and the typical flow of requests within the pipelines.

For more information about the individual projects, see the links below.

- EUCOMM http://www.eucomm.org
- KOMP http://www.komp.org
IKMC Web portal

The IKMC website (http://www.knockoutmouse.org) functions as a portal, displaying information about available knockout resources together with additional genomic information.

Inspecting Apoe IKMC available resources.

To explore the ongoing targeting projects for Apoe, search for Apoe using http://www.knockoutmouse.org. Click on the “Details” link as shown in the arrow below.

This results in the running projects view for Apoe, with information about specific projects for the gene, statuses and reagent availability with links to the repositories for ordering. More information about the strategy and targeting vector can be displayed, by clicking the 'Allele Details' link.
Explore the details available for CSD, TIGM and Regeneron

(Nb. TIGM provides links to NCBI with the traps sequence and information)
IKMC Portal Pipeline Statuses

When IKMC projects are displayed, they are represented as a series of bars which are coloured blue until the last successful step. For example, the most successful CSD(KOMP/EUCOMM) project for the ApoE gene is represented below:

Each blue block represents the gene’s successfully passing a particular step in the KOMP/EUCOMM pipeline.

In the case of the Ccdc3 locus, the last box is shown in orange, meaning the project has not been successful at a step, in this case the ES cells phase. The reason for the failure is displayed in the orange box, in this case, “ES Cells-No QC Positives”

It is important to remember that each PROJECT corresponds to a particular case of DESIGN and VECTOR CASSETTE and is not unique or exhaustive for efforts to target a particular gene. So even if a status is “project terminated”, it usually means that a particular approach to target a gene has been abandoned and another one has commenced (through a new design and/or vector), not that all efforts at targeting the gene in question have ceased.
A list of the various IKMC KOMP(CSD)/EUCOMM statuses with explanations are shown below grouped by the stage of the most advanced pipeline product:

**EUCOMM/KOMP PROJECT STATUSES:**

<table>
<thead>
<tr>
<th>Status</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mice - Genotype confirmed</strong></td>
<td>The genotype of testcross germline offspring has been confirmed molecularly.</td>
</tr>
<tr>
<td><strong>Mice - Germline transmission</strong></td>
<td>Test crosses of chimaeras indicate germline transmission of the ES cell mutation based on coat color.</td>
</tr>
<tr>
<td><strong>Mice - Microinjection in progress</strong></td>
<td>Targeted ES cells have been microinjected for the purpose of creating mutant mice for distribution.</td>
</tr>
<tr>
<td><strong>ES Cells - Targeting Confirmed</strong></td>
<td>Correctly targeted ES cell clones have passed LR-PCR/sequenced-based QC. These ES cell clones can be requested from the EUCOMM/KOMP repositories.</td>
</tr>
<tr>
<td><strong>ES Cells - Targeting Unsuccessful Project Terminated</strong></td>
<td>No targeted clones have been obtained and the project has been terminated.</td>
</tr>
<tr>
<td><strong>ES Cells - No QC Positives</strong></td>
<td>ES cell clones were picked and screened, but did not pass LR-PCR/sequenced-based QC. These ES cell clones may be re-screened with additional genotyping primers.</td>
</tr>
<tr>
<td><strong>ES Cells - Electroporation Unsuccessful</strong></td>
<td>Electroporation performed, but yielded few or no colonies</td>
</tr>
<tr>
<td><strong>ES Cells - Electroporation in Progress</strong></td>
<td>Electroporation of the final targeting construct is underway.</td>
</tr>
<tr>
<td><strong>Vector - Vector Complete</strong></td>
<td>A final targeting vector (post Gateway) has been constructed, has passed sequence-based QC, and is available for use in ES cells. These vectors can be requested from the EUCOMM/KOMP repositories.</td>
</tr>
<tr>
<td><strong>Vector - DNA Not Suitable for Electroporation</strong></td>
<td>A final targeting vector (post Gateway) has been constructed, has passed sequence-based QC, but has failed gel-based QC prior to electroporation (low DNA yield, incomplete digestion or multiple DNA bands). These vectors will be re-prepped for electroporation and can be requested from the EUCOMM/KOMP repositories.</td>
</tr>
<tr>
<td><strong>Vector - Vector Complete Project Terminated</strong></td>
<td>A project which has targeting vector but after re-synthesis still no successful ES cell, so it was terminated. Also vectors constructed but not optimal for targeting the gene of interest.</td>
</tr>
<tr>
<td><strong>Vector - Vector Unsuccessful - Project Terminated</strong></td>
<td>A project is terminated when two attempts at producing a valid conditional design (allele) have failed.</td>
</tr>
<tr>
<td><strong>Vector - Vector Unsuccessful Alternate Design in Progress</strong></td>
<td>The vector for a failed targeting project is in the process of being re-designed</td>
</tr>
<tr>
<td><strong>Vector - Vector Initial Attempt</strong></td>
<td>The construction of a final targeting construct, following</td>
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### The Bioinformatics of Mutant Mouse Resources

#### Module 3: IKMC Resource

<table>
<thead>
<tr>
<th>Status</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unsuccessful</strong></td>
<td>the Gateway exchange reaction, was not successful or did not pass sequence-based QC; pending a second attempt.</td>
</tr>
<tr>
<td><strong>Vector - Vector Construction in Progress</strong></td>
<td>Oligos for recombineering have been ordered for the construction of the targeting vectors with vector construction to follow</td>
</tr>
<tr>
<td><strong>Design - Design Completed</strong></td>
<td>The gene has been manually annotated by the Havana group and the design of a conditional allele has been generated.</td>
</tr>
<tr>
<td><strong>Design - Alternate Design Requested</strong></td>
<td>The gene's earlier designs have not succeeded, and a new design is needed.</td>
</tr>
<tr>
<td><strong>Design - Design Not Possible</strong></td>
<td>The gene has been manually annotated by the Havana group but the design of a conditional allele has not been possible. Reasons for this may include the absence of frame-shifting (critical) exons, the introns flanking critical exons are too small, too few exons (our vector design strategy requires the presence of at least two exons), etc. Requests are usually put on hold in this status unless other designs (e.g. deletion alleles) are specified. Many of these genes will become conditional targets in the EUCOMMTOOLS program</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Status</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-Pipeline - VEGA Annotation Requested</strong></td>
<td>The first step in the process of making an allele is to manually annotate the gene. This is carried out by the Havana group and the annotation is made publicly available on the Vega website at the next release.</td>
</tr>
<tr>
<td><strong>Pre-Pipeline - Withdrawn From Pipeline</strong></td>
<td>The EUCOMM/KOMP project(s) will not, or has ceased to, work on this gene. This may occur for a number of reasons for example if it is registered as having 6 or more hits in the TIGM database.</td>
</tr>
</tbody>
</table>

#### The Regeneron pipeline comprises the following statuses:

- Regeneron Selected
- Parental BAC Obtained
- Design Finished/Oligos Ordered
- Targeting Vector QC Completed
- Vector Electroporated into ES Cells
- ES cell colonies picked
- ES cell colonies screened / QC no positives
- ES cell colonies screened / QC one positive
- ES cell colonies screened / QC positives
- ES Cell Clone Microinjected
- Germline Transmission Achieved
Conditional Knockout Allele

Both EUCOMM and KOMP-CSD use a conditional targeting knockout allele, using the strategy below.

When exposed to the site-specific recombinases, Cre and Flp, the KO-first allele (tm1a) can produce reporter knockouts (tm1b), conditional knockouts (tm1c), and null alleles (tm1d).

The strategy followed by current international pipelines to produce conditional knockouts, is based on the 'knockout first' allele (Testa et al., 2004). This strategy is implemented by first choosing a critical exon or exons (CEs) which, when deleted, would create a null allele. Selected CEs are ideally in the first coding half of the gene, which when deleted induces a frameshift that avoids reinitiation (Kozak, 2001) and disrupts protein domain structure. Introns flanking the critical exon(s) must have a minimum size (~500bases) to comfortably accommodate the cassettes, which are inserted at given distances (300bp upstream, 100bp downstream) to avoid interfering with the exonic splicing signals. The first allele to be generated is a reporter-tagged knockout allele (Knockout-first, tm1a) arising from the insertion of an IRES/lacZ – promoter-driven Neo targeting cassette upstream of the CE. The cassette is flanked by FRT sites, so that a conditional allele (tm1c) can be generated after treatment with Flp recombinase. This conditional allele can then be exposed to Cre recombinase, deleting the loxP flanked CE, to produce a null allele (tm1d). This strategy is particularly useful in long multi-exon genes. However, not all gene structures can be targeted with this approach.
Three types of allele are available from the EUCOMM and KOMP programs, listed below in order of preference:

**Conditional frameshift alleles.** In this allele the eventual deletion of the critical exon (CE) would induce a frameshift in all expressed products. In most cases the frameshift results in nonsense mediated decay (NMD) due to the presence of a premature termination codon (PTC) upstream from splicing signals (Lejeune &. Maquat 2005).

**Conditional domain-disruption alleles.** In this case the deletion of the CE would not induce a frameshift: the product would not be subject to NMD and hence would likely produce a protein, but the CE is chosen such that a significant part of the protein’s functional domain is deleted.

**Deletion alleles (KOMP only)** are non-conditional and are designed when none of the above is possible.

Although comprehensive, not all genes in the mouse genome are amenable for targeting using these design strategies. Single exon genes and genes with the majority of the coding sequence in the first exon can not be targeted through these strategies. These genes will be targets of conditional approaches involving the use of artificial introns as part of the EUCOMMTOOLS program.

**Vector Construction/Modularity**

Background knowledge of the pipelines and processes which are involved in the production of targeting vectors and targeted ES cells can be extremely useful in understanding their properties and potentialities. What follows is a description of the pipeline used to make conditional targeting vectors for the EUCOMM and KOMP-CSD programs. Full details of the pipeline and the technology used in the production of conditional IKMC targeting alleles is provided by Skarnes et al. (2011).

A modular strategy is employed in vector construction, which enables vector intermediates to be recycled to make other useful alleles (eg fluorescent reporters or recombinase knock-ins). These approaches figure prominently in the new EUCOMMTools program which aims to extend the value and utility of the EUCOMM/KOMP CSD vector resources.
High Throughput Targeting Pipeline

The three major phases of the EUCOMM/KOMP pipeline are outlined below:

### I) *In silico* Phase

<table>
<thead>
<tr>
<th>PROCESS</th>
<th>STEP</th>
<th>RESOURCES GENERATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual gene annotation / Automated vector design</td>
<td>Vector Design</td>
<td>Vega gene structures / Conditional designs</td>
</tr>
<tr>
<td>96-well serial liquid recombineering</td>
<td>BAC recombineering</td>
<td>Library of intermediate plasmids</td>
</tr>
</tbody>
</table>

### II) Vector Construction Phase

- 3-way Gateway exchange reactions
- Final vector assembly
- Library of final targeting vectors

### III) ES cell Gene Targeting Phase

- 25-well parallel electroporations of C57BL/6 ES cells
- ES cell production
- Long-range PCR genotyping and sequencing
- LacZ-tagged conditional mutant ES cell clones

Current High Throughput Targeting Pipeline production numbers are displayed on the IKMC homepage and are updated daily.

<table>
<thead>
<tr>
<th>Total Generated Products</th>
<th>EUCOMM</th>
<th>KOMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>570</td>
<td>304</td>
</tr>
<tr>
<td>ES Cells</td>
<td>6371</td>
<td>4956</td>
</tr>
<tr>
<td>Vectors</td>
<td>7858</td>
<td>6639</td>
</tr>
</tbody>
</table>

These numbers will grow to over 13,000 targeted ES cell lines by the end of 2011 and should eventually include ALL GENES in the future. The EUCOMMTOOLs grant will extend the collection of conditional mutations to 3,000 new genes using new approaches and technologies.
The starting point for vector construction are libraries of indexed (end-sequenced) mouse genomic BACs (Bacterial Artificial Chromosomes). BACs are very large (75-250 kb genomic DNA insert) vectors which are very stable and present at a single copy per bacterial host cell. The C57Bl/6 vectors we make in EUCOMM/KOMP are derived from C57Bl/6 RPC23-24 libraries. C57Bl/6 is the reference strain for the mouse genome sequence, thus simplifying recombineering oligo design. Use of end-sequenced, indexed BAC libraries obviates the need for complicated screening strategies to identify genomic clones of interest, which had previously been one of the most difficult steps in targeting vector construction. A highly efficient, arabinose inducible recombineering plasmid is then introduced into the BAC host to make a recombineering ready system, with no need the need to transfer the BAC to another host.

An arrayed set of BACs for 96 genes is ordered from the repository and becomes the substrate for three serial recombineering steps. The first two steps are insertions in cis into the single copy BAC, the third is a gap repair reaction to sub-clone the modified region of the BAC with DNA homology arms into a plasmid vector. The recombineering reactions are carried out in the liquid phase with no analysis of single bacterial colonies until the end of the process. This strategy is the result of both the exquisite specificity of recombineering and the negligible background obtained. In essence, the vector construction process transforms a set of 96 genomic BACs into a set of 96 conditional plasmid gene targeting vectors as summarized below:

**Targeting Vector Construction Pipeline**

![Targeting Vector Construction Pipeline Diagram]

- 96 Indexed BAC Clones
- 96 Plasmid sized Conditional Targeting Vectors
- 96 Well RECOMBINEERING
- KEY FEATURES:
  - Serial Liquid Transfer
  - 96 well electroporation
  - Gateway based modularity
  - Compatibility with indexed BAC resources

EUCOMM/KOMP plasmid targeting vectors employ modular switch systems based on Invitrogen’s Gateway™ technology. Gateway is a simple in vitro DNA transfer system based on variants of phage lambda site specific recombination sites and purified recombinase proteins. Two heterospecific Gateway switch elements are employed, one (B1/B2) being used to insert the gene targeting element upstream of the critical exon region, and another (B3/B4) to place a negative selection marker on the vector and switch to a high copy number plasmid backbone. The Gateway elements not only lend
modularity to the system, but also offer a number of practical advantages in the vector construction process. The technology is also simple and accessible and has been employed in a number of other large, genomic scale resources.

The particular conditional allele engineered by each targeting vector is a function of the specific oligonucleotide sequences used in the recombineering of the two insertions and the gap repair. This defined configuration of oligo sequences is referred to as a “design”, which is centered on a “critical” exon or exons whose removal either disrupts the reading frame of the gene or deletes a protein domain. Designs are created with a fixed set of criteria, namely targeting arms of about 5kb and a deleted “critical exon” region of ideally 2kb or less. An example of a design with candidate regions for recombineering oligo designs highlighted is shown below:

A computer program called AOS (Array Oligo Selector) then picks optimal oligos in the candidate regions as shown below. The exact position of these six oligonucleotides fixes the design. These six oligos are shown superimposed below:

The design can also be viewed in a genome browser such as ENSEMBL, displaying the positions of the six recombineering oligos, which will be discussed in more detail later.
The oligonucleotides used in the recombineering are 70mers, the 20 most 3' nucleotides (often referred to as “appending sequences”) are used to amplify common “anchor” sequences in the cassettes, the 50 5' most nucleotides serve as regions of homology for the recombineering process. The oligos are ordered on 96 well plates in identical arrays to the mouse BACs they are meant to target. Two plates of matched oligos are mixed with a standard template which varies with the specific step and used in a high fidelity PCR reaction. The set of 96 PCR products is then desalted by ultrafiltration and used for electroporating the matched arrayed set of BAC containing E.Coli after the cells have been induced to produce recombineering proteins with arabinose. This process is outlined below:

A Step-wise diagram of the 96 well vector recombineering pipeline is shown below:

![PCR Generation of Gene Specific Recombineering Fragments](image)
The specific steps of the process of building a set of 96 targeting vectors are as follows:

**Step 1 Bac transformation**
Introducing the pSC101 based, arabinose induced recombineering plasmid pSC101gbaA(1) into the BAC carrying E. Coli clones from indexed libraries.

**Step 2 “U” (upstream) Insertion**
Insertion into the BAC of a cassette containing Gateway R1 and R2 sites with bacterial positive (zeocin) and negative (PheS) (5) selection markers. This site, in an intron upstream of a “critical” exon (one whose removal disrupts the gene’s reading frame) is later used as the point of Gateway mediated insertion of various gene targeting cassettes (eg reporter-promoter-neo) and the 5’ loxP site for the conditional allele.

**Step 3 “D” (downstream) Insertion**
Insertion into the BAC of a floxed KanR cassette downstream of the critical exon. This occurs in cis on the single copy BAC and will be the site of the 3’ loxP site for the conditional.

**Step 4 “G” Gap Repair Rescue**
Gap repair rescue sub-cloning of the region of the BAC with the critical exon and two previous engineered insertions flanked by homology arms of approximately 5kb on each side. The Gap repair backbone is a linearized pBR322 based plasmid. This backbone also contains heterologous R3 and R4 Gateway sites which allow the backbone to be switched to a high copy number version containing negative selection for ES cell targeting (PGK-DTA in our pipeline).

**Step 5 Cre Transformation**
Plasmid DNA is purified and transformed into bacteria containing a pSC101 plasmid which has been pre-induced to contain large amounts of cre protein (by heat, ts lambda Pr promoter). This step both purifies the correct gap repair product away from artefactually re-circularized backbone and reduces the floxed cassette from the D insertion step into the single 3’ loxP site.

The resulting plasmid is referred to as an “intermediate vector” which can then be converted to any number of “final vector” alleles via the Gateway modular system.

For quality control, we initially sequenced all intermediate vectors across all six recombineering junctions in EUCOMM/KOMP. Designs which fail initial recombineering are “re-designed” with a new set of oligonucleotides with a success rate of over 80%
Introduction to Gateway Mediated Modular Targeting Vector Assembly

Final Gene Targeting Vectors are assembled from three component plasmids (shown below) using a Gateway based system of modular replacement based on a modified system of λ phage site specific recombination in vitro. More information on the Gateway system can be obtained at Invitrogen’s website (www.invitrogen.com). Gateway sites employed in EUCOMM/KOMP vectors are of two complimentary varieties, L site and R sites. L and R sites have specificities designated by a number, eg. L1 and R2. L sites react with R sites of identical specificity but not with other Gateway sites, eg L1 with R1 and L3 with R3 but NOT L1 with R3 or L1 with L2. The LR Gateway reaction leaves minimal 25bp Gateway B (specificity) sites in the final vector. The reaction is driven by a purified enzyme mix called LR Clonase (available from Invitrogen) and involves a simple in vitro incubation followed by a bacterial transformation step. Gateway involves no PCR and is therefore non-mutagenic.

EUCOMM/KOMP vector resources contain TWO Gateway based switching systems, each consisting of a set of two Gateway sites. Intermediate targeting vectors contain in total FOUR Gateway sites (R1/R2 and R3/R4) which constitute these two independent switching systems.

The first system is used to introduce a gene targeting element upstream of the critical exon and employs R1-L1 and R1-L2 Gateway sites. The R1 and R2 sites flank a negative selection marker (pheS, a conditional negative selection marker) on the intermediate vector plasmid (ampR, ZeoR) and the
L1 and L2 sites flank a gene targeting element (eg. En2SA–IRES-LacZ-promoter-neo-pA) on a second targeting element plasmid (ClonNATR).

The second system is used to switch the gene targeting vector component (homology arms plus gene targeting element) to a new high copy number plasmid backbone containing a mammalian negative selection gene (DTA) and employs R3-L3 and R4-L4 Gateway sites in an “inverted” orientation. The R3 and R4 sites are located at the ends of the homology arms of the gene targeting vector and the L3 and L4 sites flank a negative selection marker (ccdB) on the new plasmid backbone (KanR or SpecR) which also contains a mammalian negative selection cassette.

We have found that these steps can be efficiently combined into a single reaction containing three component plasmids:

-“Intermediate targeting vector” plasmid with R1/R2 and R3/R4 Gateway sites. The intermediate targeting vector is gene specific and contains the homology arms, targeting cassette and AsiSI vector linearization site. It can be used to construct many different potential final targeting vectors in a modular fashion and one of the resources generated by KOMP/EUCOMM.

-Gene targeting element plasmid with L1 and L2 Gateway sites. This will be referred to as “L1L2 plasmid”. The EUCOMM/KOMP programs employ mainly promoter containing or promoterless lacZ reporter, conditional allele vectors. L1L2 cassettes for constructing other types of knock-in alleles such as fluorescent reporters or recombinases are being developed as part of the EUCOMMTOOLs program.

-High copy number plasmid backbone containing a DTA negative selection cassette with L4 and L4 Gateway sites. This will be referred to as “L3L4 plasmid”.


A diagram of the three-way Gateway reaction and the final targeting vector obtained is shown below:

**3-way Gateway reaction**

![Diagram of the three-way Gateway reaction](image)

**Modular Gateway Applications of EUCOMM/KOMP Vectors**

Although the EUCOMM/KOMP programs have been making standard lacZ conditional alleles, the modular Gateway vector system can be used in the future to make any number of different useful alleles, as shown below:

![Modular targeting cassettes for the generation of other useful alleles](image)

A L1L2 Gateway targeting cassette developed for a single application immediately becomes applicable to *ALL GENES* with EUCOMM or KOMP Gateway adapted intermediate vectors.
The recently funded EUCOMMTOOLS grant has provided support for constructing a set of vector module resources of importance to the mouse genetics community. The basic deliverables will start with fluorescent reporter cassettes (H2bVenus, H2bCherry) and site-specific recombinases (Cre-ERT2, Flp, Dre etc) but can extend to any project which results in the building/testing of cassette modules with broad interest to the community. These cassettes have been built with a modular design which facilitates the building of new variants. The basic promoter containing promoterless cassettes for EUCOMMTOOLS are shown below:

The 5’ Frt and 3’ loxP sites of these cassettes also enable their use in a dual RMCE (dRMCE; recombination mediated cassette exchange) system (Osterwalder et al, Nature Methods 2010). The RMCE system is relevant to cases where there is already a correctly targeted ES cell line for the gene of interest. If there is not a targeted cell line, but there is an intermediate targeting vector, a new targeting vector can be assembled with the EUCOMMTOOLS L1L2 cassette plasmid, the intermediate vector plasmid and an L3L4 DTA backbone plasmid. The dRMCE system involves transient electroporation with two plasmids, a dual iCre/FlpO expression plasmid(pDIRE) and a replacement plasmid containing the new allele(in the illustration a fluorescent reporter; pDREV) with a different antibiotic resistance marker. dRMCE can be employed to switch the reporter allele in a previously targeted cell line and involves only simple short range PCR genotyping.
The *in vivo* reactions involved below are illustrated below:

An example of the 3 plasmid Gateway mediated assembly of an H2bVenus reporter vector from gene specific intermediate vector and components is shown below. Here the goal is to build a new targeting vector with a different reporter:
Some of the existing “flavors” of L1L2 vector cassettes are illustrated below, with a “Standard” lacZ conditional cassette shown at top:

Intermediate targeting vectors can be recycled for a number of diverse purposes, a particularly interesting one has been the development of separate 1st and 2nd allele vectors for constructing conditionally homozygous ES cells for in vitro investigations (Tate and Skarnes, Methods 2011) In this case same intermediate vector is used to create two separate gene targeting vectors, one conditional and one null (with two different antibiotic resistance genes).