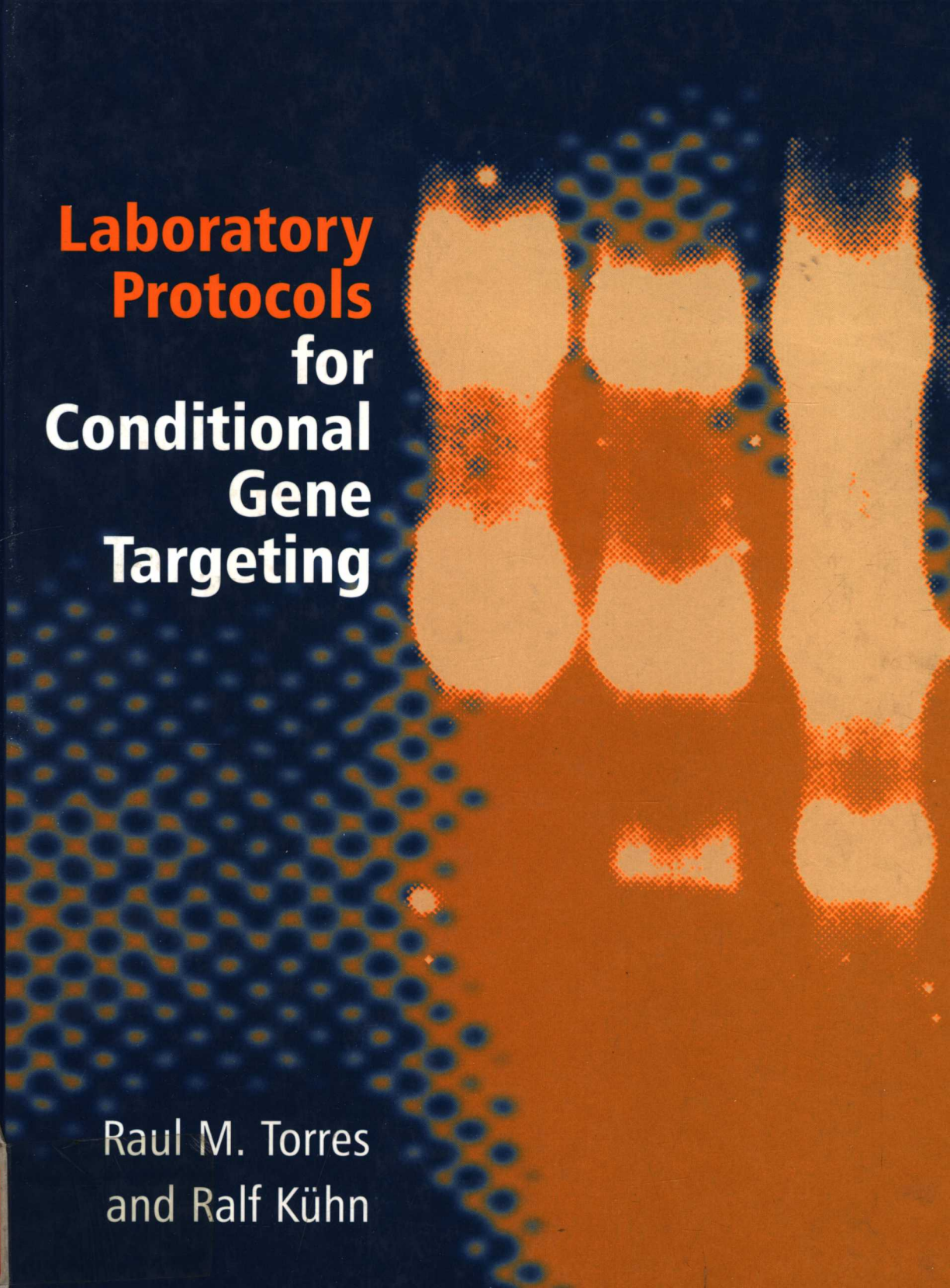
Laboratory Protocols for Conditional Gene Targeting



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Foreword

This book grew out of Raul Torres' initiative to put together our lab protocols on gene targeting, so that newcomers would have a solid basis for starting their work. This became mandatory in particular when, due to Hua Gu's efforts, conditional gene targeting became feasible and many students and postdocs in the lab began to work in this area. When I saw Raul's text, which came as a surprise, and noticed the lively and encouraging style in which he was able to write, I immediately suggested he consider publication. At this point Ralf Kühn, member of our initial gene targeting team and the first to achieve inducible gene targeting, joined the venture, and the focus of the book became conditional gene targeting mediated by the Cre/loxP system.

I am delighted that the book is now ready for publication and think that it comes at the right time. Conditional gene targeting is just getting off the ground, and, offering ultimately to introduce mutations into any predetermined gene of the mouse in any cell type at any time of development, has a broad perspective. For all those who want to use this new tool the book should be useful in daily life. Let us hope that this approach will lead to novel insights into how vertebrate genes control biological functions in vivo!

January 1997, Klaus Rajewsky

"...anyone who leaves behind him a written manual, and likewise anyone who receives it, in the belief that such writing will be clear and certain, must be exceedingly simple-minded..."

Plato, Phaedrus

Preface

Embryonic stem (ES) cells retain the pluripotency of early embryonic-derived cells and, as such, have the potential to direct the development of a mouse. Coupled with the ability to identify homologous recombination events within these cells, the ability to manipulate ES cells has revolutionized the way we are able to approach biological questions. For this reason, the work of the many embryologists, cell biologists, and biochemists who pioneered these techniques deserve special recognition in making gene targeting the powerful technique that it has become today.

Indeed, from the first report of gene targeting in murine ES cells by Thomas and Capecchi (1), an impressive progress in the analysis of gene function in the mouse has been achieved through numerous mutants (2-4). As a result, the popularity of gene targeting is still increasing as many investigators turn to this technique to address their particular biological question (4-8). There has been, however, little variation on the strategy of how gene targeting vectors are constructed and on the type of genetic alterations generated by them.

A minimal generic targeting vector used for gene inactivation or modification will generally possess a region of homologous sequence juxtaposed to a selectable marker. Variations on this basic structure depend on whether genetic material is to be inserted or replaced during the homologous recombination event (1, 5, 6, 9). The end result with either of these strategies is usually the presence of the selectable marker at the targeted locus (simply inserted or replacing endogenous genetic material), which inactivates gene function. Several sophisticated variations of this general strategy, referred to as 'hit-and-run' and 'double replacement' (see below) have been established which aim to introduce subtle mutations or minimize the perturbations generated at the locus of interest.

To achieve the same purpose, in Klaus Rajewsky's unit at the Institute for Genetics in Cologne, Hua Gu pioneered an alternative strategy which exploits the bacteriophage Cre/loxP recombination system in combination with existing ES cell technology. In addition to the generation of subtle mutations, this system allows for a number of other genotypic options in ES cells by strategically incorporating loxP sites into the targeting vector, and subsequent expression of Cre recombinase, *after* homologous recombinants have been identified. Furthermore, when Cre is expressed in transgenic mice (and crossed to a loxP-containing target gene) the desired gene modification can be made conditional based on the expression properties of the promoter region used to drive Cre expression. The

usefulness of this technology has already become apparent as a valid approach for the analysis of in vivo gene function. The numerous applications of the Cre/loxP recombination system significantly expands the potential of gene targeting methods (10-13) and should further stimulate genetic analysis in the mouse.

This book is not necessarily intended to be a replacement for the existing useful guides on embryonic stem cells and gene targeting (14-16). Rather, with this book we have attempted to summarize the capabilities of, and our experience with, the Cre/loxP recombination system as used together with gene targeting. The protocols found in this manual are a collection of bench-tested protocols which we have adopted for gene targeting in our lab and with particular emphasis on using the Cre/loxP recombination system. Our hope is that the many newcomers to gene targeting, as well as the experienced researchers in this field, will find this advice valuable.

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Abbreviations

bp	Basepairs
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylfulfoxide
DNase	Deoxyribonuclease
EF	Embryonic fibroblasts
ES	Embryonic stem (cells)
FCS	Fetal calf serum
G418	Geneticin
GANC	Gancyclovir
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
i.p.	Intraperitoneal
IU	International units
kb	Kilobasepairs
LIF	Leukemia inhibitory factor
mmc	Mitomycin c
MT-PBS	Mouse isotonicity PBS
neo	Neomycin resistance gene
PBS	Phosphate-buffered saline
p.c.	Post coitus
PCR	Polymerase chain reaction
(HSV)-tk	(Herpes simplex virus) thymidine kinase
SDS	Sodium dodecyl sulfate

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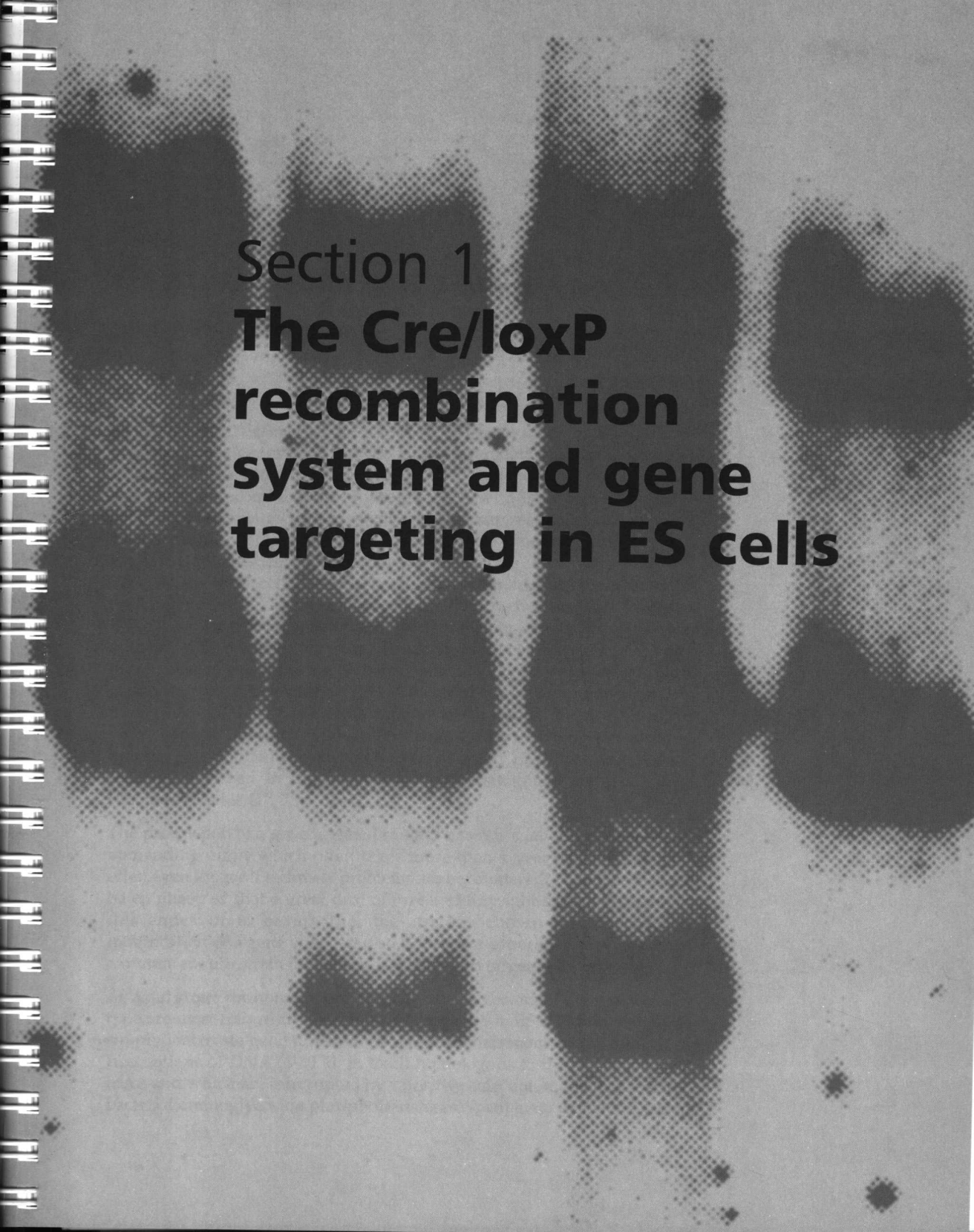


Section 1
The Cre/loxP recombination system and gene targeting in ES cells

Chapter 1
Gene targeting strategies

Gene targeting, defined as the introduction of site-specific modifications into the mouse genome by homologous recombination, is generally used for the production of mutant animals to study gene function in vivo. Since homologous recombination of foreign DNA with endogenous genomic sequences is a relatively infrequent event in mammalian cells, compared to its random integration, the only efficient gene targeting method presently established utilizes pluripotent murine embryonic stem cell lines. Using these cells, the selection of rare, homologous recombinant ES cell clones in vitro can be accomplished. When such genetically modified ES cells are introduced into a preimplantation embryo they can contribute, even after extensive in vitro manipulation, to all cell lineages (including germ cells) of the resulting chimeric animal. The breeding of germline chimeras, which transmit an ES cell derived mutant chromosome(s) to their progeny, allows the establishment of an animal heterozygous for your genetic alteration and, importantly, by further breeding a homozygous mutant mouse strain. The necessity of chimera production may be bypassed in the future when spermatogonial stem cell lines become available (17, 18).

The production of a gene targeted mutant strain is a laborious, technically demanding effort which often takes more than a year to complete, and often even longer if technical problems are encountered. Therefore, it must be emphasized that a great deal of care and foresight should be given to this endeavor to ensure that the strategy chosen for the targeted modification of a gene will later also fulfill your experimental needs when a mutant mouse strain has been generated from targeted ES cells.

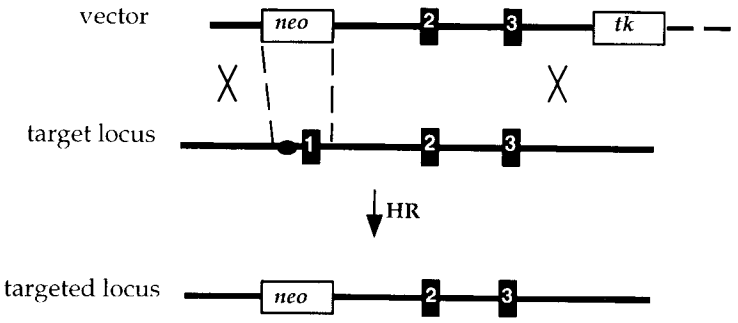
As a substrate for homologous recombination, vectors of the replacement type are most frequently used for gene targeting in ES cells and usually to simply inactivate gene function. A typical replacement vector consists of two regions of DNA (4-10 kb in total) homologous to the genomic target locus and which are interrupted by a positive selection marker such as the bacterial aminoglycoside phosphotransferase (neo) gene which is selected

for with G418. This marker is either inserted into the homology region or, alternatively, replaces genomic sequence located between the homology arms (Figure 1a). If homologous recombinant ES clones will be identified by PCR one of the vector arms must be kept relatively short (1-2 kb) to ensure efficient amplification. A thymidine kinase (tk) gene is often included at the end of the long homology arm of the vector and serves as an additional negative selection marker (using gancylovir) against ES clones which have randomly integrated the targeting vector. Thus, homologous recombinants can be enriched by both positive and negative selection (19). The positive selection marker is primarily used to enrich for the rare stably transfected ES cell clones (obtained at a frequency of about one in 10^4 cells by electroporation) but frequently also serves as a mutagen, if appropriately designed, by insertion of the marker gene into a coding exon or the replacement of coding exons of the target gene by the marker. In addition, a small, nonselectable (e.g. point) mutation can be introduced into the homology region of the vector which is cotransferred into the target locus together with the selection marker in a fraction of homologous recombination events. In any case, the end product of a targeting experiment using a replacement type vector which includes a positive selection marker is the presence of the selection marker gene in the targeted locus which cannot be further modified. Hence, this gene targeting strategy is only suitable for the generation of a nonfunctional (knock out) allele of a target gene but should not be used for the introduction of subtle mutations since the selectable marker may disturb the regulation and splicing of the modified gene (20, 21). However, this precaution does not exclude, even if the target gene should be simply inactivated, possible interference of the selectable marker with genes adjacent to the target gene or coded on the opposite strand (22-24). Moreover, when using the positive selection marker to inactivate gene function, careful consideration should be given to potential outcomes besides the desired gene inactivation. These would include alternative splicing of the affected exons (25) and/or the potential generation of dominant negative molecules.

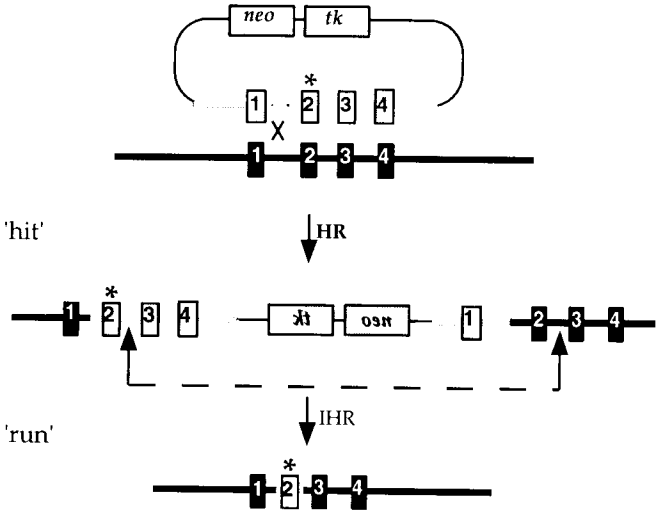
To circumvent the problems associated with the presence of a selection marker in a targeted locus several two-step gene targeting strategies have been developed which allow the introduction of subtle, nonselectable mutations into, and the removal of the selectable marker from, the targeted gene. The 'hit-and-run' method (9, 26, 27) also termed 'in-out' (28), utilizes an insertion type targeting vector which is linearized for transfection within the homology region of the target gene. In the first targeting step ('hit'), homologous recombination leads to the complete integration of the vector into the target locus generating a partial duplication interrupted by plasmid sequences and selection markers (neo as positive and tk as negative marker; Figure 1b). In the example shown in Figure 1b a point mutation was introduced in exon 2 of the homologous sequence contained within the targeting vector. The duplication can be resolved by an *intra*chromosomal homologous recombination event or

unequal sister chromatid exchange ('run') occurring spontaneously at a low rate in a population of targeted ES cells. Such (tk-negative) cells can be enriched from the tk-positive majority using selection (e.g. gancyclovir) which kill tk-expressing cells. If recombination occurs as depicted in Figure 1b the point mutation in exon 2 is incorporated into the restored target gene while all heterologous sequences are lost from the targeted locus. However, the intrachromosomal recombination event can also result in the restoration of the wildtype allele depending on the actual point of exchange. For a detailed account of hit and run targeting vector construction and identification of targeted clones see (27).

a) Gene inactivation



b) Hit and run



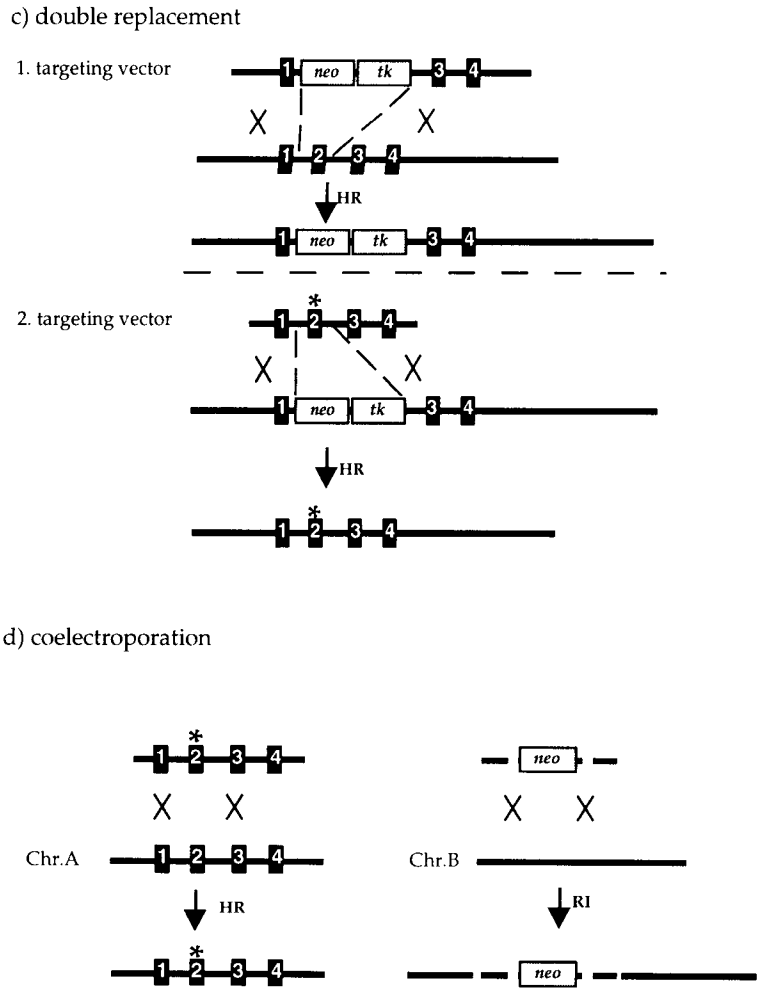


Figure 1. Conventional gene targeting strategies. Cre/loxP based gene targeting is depicted in Figures 4-12. Symbols are as indicated in Figure 4. Extrachromosomal recombination products are not shown. **(a)** Simple gene inactivation with a replacement type vector. The promoter and first exon (filled rectangle) of the target gene are exchanged by a neomycin resistance gene (neo) by homologous recombination (HR) with the linearized targeting vector. Crossover points are depicted by an X. The tk gene and plasmid sequences (thick stippled line) are lost during homologous recombination. **(b)** Introduction of a point mutation by the 'hit-and-run' method. The integration type targeting vector, containing neo and tk genes within the plasmid backbone (thin line), is linearized within the homology region of the target gene (shaded lines and rectangles) between exons 1 and 2 (rectangles). A point mutation (asterisk) is introduced in exon 2 of the targeting vector. In the first (hit) step the target locus (solid black line and rectangles) is