Haplobank

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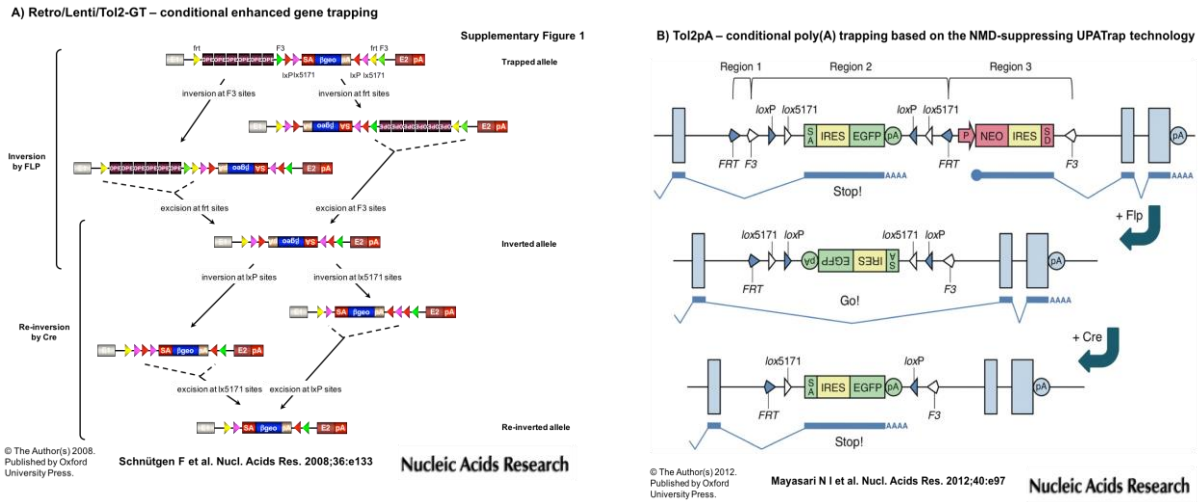
PCR STRATEGY TO CONFIRM THE INVERSION OF THE SPLICE ACCEPTOR UPON CRE/FLP EXPRESSION

When inserted into an intron of an expressed gene, the gene traps are transcribed from the respective endogenous promoter of the gene, yielding fusion transcripts in which the upstream exons are spliced to βgeo of the gene trap cassette. Since transcription is terminated prematurely at the inserted polyadenylation site, the processed fusion transcript encodes a truncated and nonfunctional version of the cellular protein plus βgeo. As loxP and frt sites flank the gene trap cassette, it is possible to reverse that KO effect by transiently infecting cells with a Cre/Flp-expressing plasmid. By this, an isogenic sister clone is generated which may serve as a perfect internal control. If required, that clone can even be reverted a second time with Flp/Cre recombinase due to the presence of frt sites, giving you various possibilities for validation. This is schematically summarized below:



In order to generate flipped/inverted sister clones, we transiently transfect cells with a Cre- or Flpo-expressing plasmid linked to a fluorphore, e.g. EGFP or DsRed (please see *Gene Trap Flipping Protocol* at https://www.haplobank.at/ecommerce/control/haplobank_resource), which can be FACS-sorted. 48h post transfection, we FACS-sort cells, collect about 300 green or red cells, and seed those cells onto a 10cm dish. 10-12d post FACS-ing/seeding, we pick several colonies (24-48 colonies), and subsequently assay them for successful inversion of the splice acceptor as described below, about 4-5d post picking.

Alternatively, if picking of single cell subclones is not required, a drug selection marker can be linked to the Cre- or Flpo-expressing plasmid. Transiently transfected cells can then be drug selected (using non-transduced cells as drug selection control) and subsequently assay them for successful inversion of the splice acceptor as described below.

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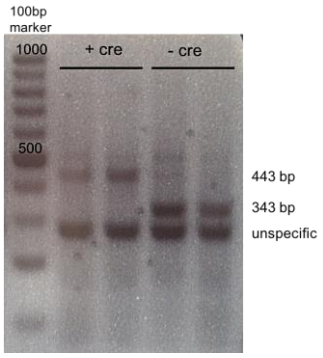
To verify the orientation of the gene trap cassette and thereby the genotype (disruptive/sense to non-disruptive/anti-sense or *vice versa*), a PCR with three primers is performed on crude ES cell lysates (prepared e.g. from 1x96w). Depending on the orientation, an amplicon bound by the 1st forward primer or bound by the inverse forward primer, respectively, is amplified.

MUTAGENISIS SYSTEM: Lenti-EGT, Retro- EGT, Tol2-EGT

GT 1st F:	TCGACCTCGAGTACCACCACACT
GT inv F	AAACGACGGGATCCGCCATGTCA
GT com R:	TATCCAGCCCTCACTCCTTCTCT



Expected bands:

- GT 1st F/GT com R: 343bps
- GT inv F/GT com R: 443bps

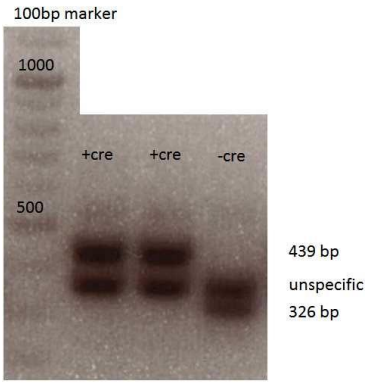
Expected band if reverted twice (1st CRE, 2nd FlpO):

- GT 1st F/GT com R: 209bps (not shown in picture)

If you flip the gene trap cassette with FlpO first, followed by Cre recombination, the length of the bands will slightly differ (10-25 bps).

MUTAGENISIS SYSTEM: Tol2polyA

Tol2 1st F	TGGGTTCAAGCGATTCTCCTGCCTCA
Tol2 inv F	AGATAGGCACCCAGGGTGATGCAAGCTC
Tol2 com R	CCGATCCATCCATCGCATATTTGGGA



Expected bands (w/o and w/CRE):

- Tol2 1st F/Tol2 com R: 326bps
- Tol2 inv F/Tol2 com R: 439bps

Expected band if reverted twice (1st CRE, 2nd FlpO):

- Tol2 inv F/Tol2 com R: 453bps

Expected band if reverted twice (1st FlpO, 2nd CRE):

- Tol2 inv F/Tol2 com R: 440bps

Please be aware that if the Tol2polyA-construct is flipped with FlpO only, the orientation cannot be determined with this PCR set-up (bands are 326bps long in both orientations).

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PCR REACTION

gDNA crude lysate	5 µl
1st F 100 µM	0.1µl
inv F 100 µM	0.1µl
com R 100 µM	0.2 µl
10 mM dNTP mix	1 µ
10x Klentaq buffer	5 µl
20x Klentaq polymerase	3 µl
dH_2O	35.6 µl
	50 µl

Biorad C1000 cycle parameters:

95deg, 3’	
95deg, 15”	35x
58deg, 20”	
72deg, 20”	
72deg, 5’	