

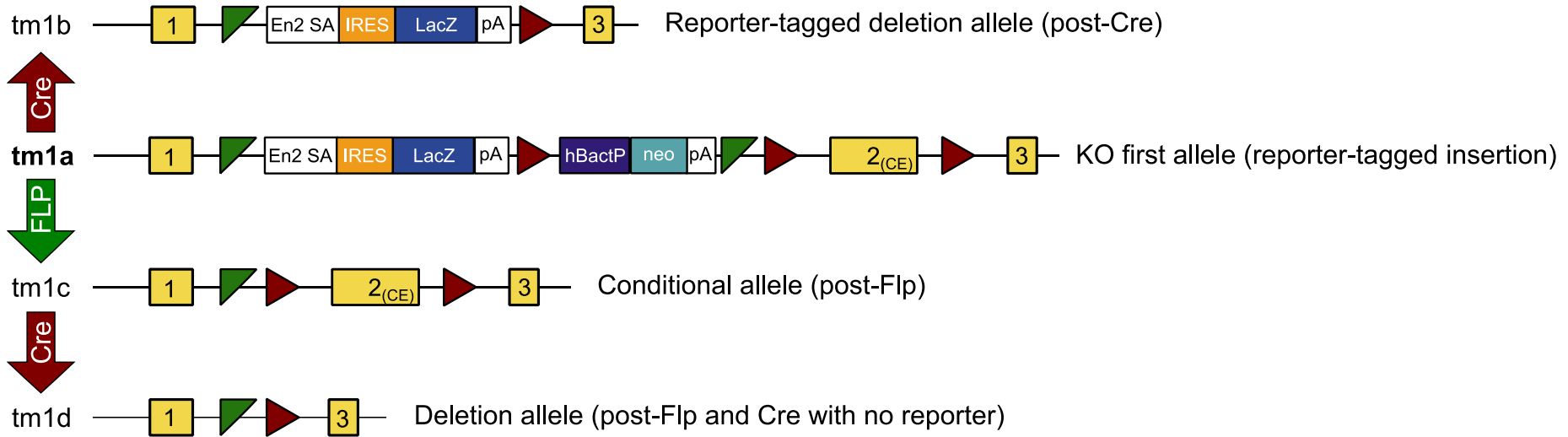
EUCOMM/KOMP-CSD allele conversion

Ed Ryder

Mouse Genetics Project

Wellcome Trust Sanger Institute

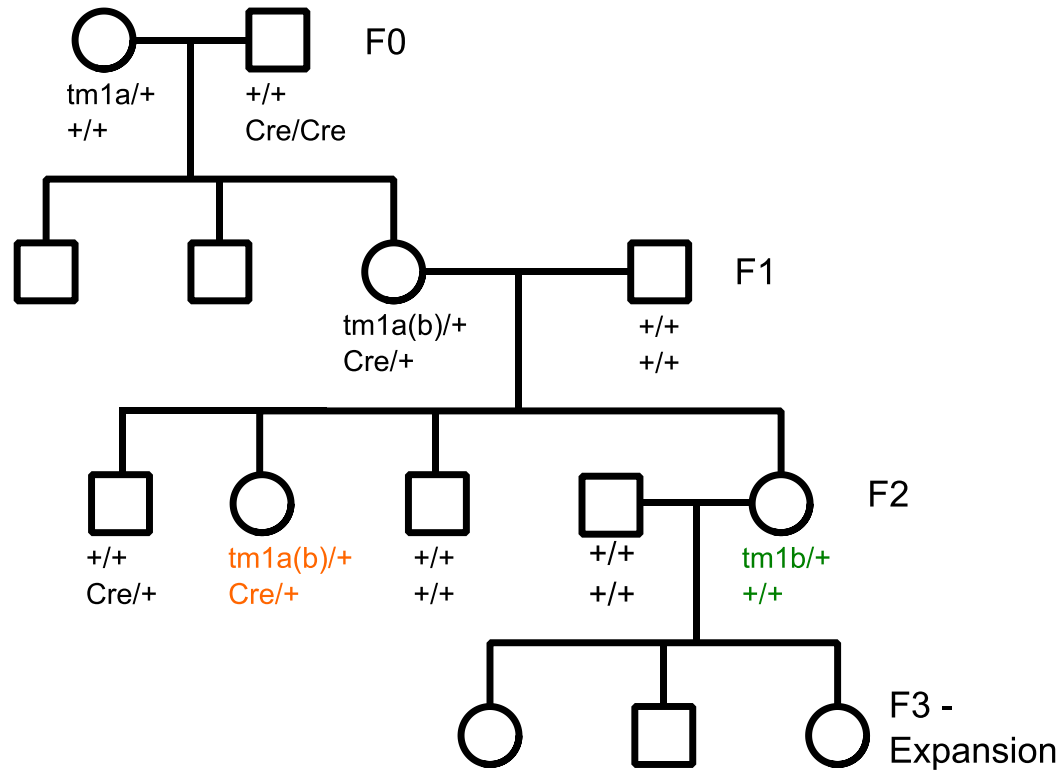
Allele types and usage



- Tm1a
 - Knockout-first relies on splice acceptor to disrupt targeted gene expression
 - Promoter-driven neo selection cassette still present
 - LacZ reporter for visualising gene expression
- Tm1b
 - Critical exon is deleted creating a frame-shift and true null
 - Promoter-driven neo selection cassette removed (promoter-driven strains only)
 - LacZ reporter for visualising gene expression
- Tm1c
 - Selection cassette including splice acceptor is removed restoring WT gene expression
 - Critical exon is floxed allowing tissue-specific deletions with a chosen Cre driver
- Tm1d
 - Deletion allele

TM1B CONVERSION

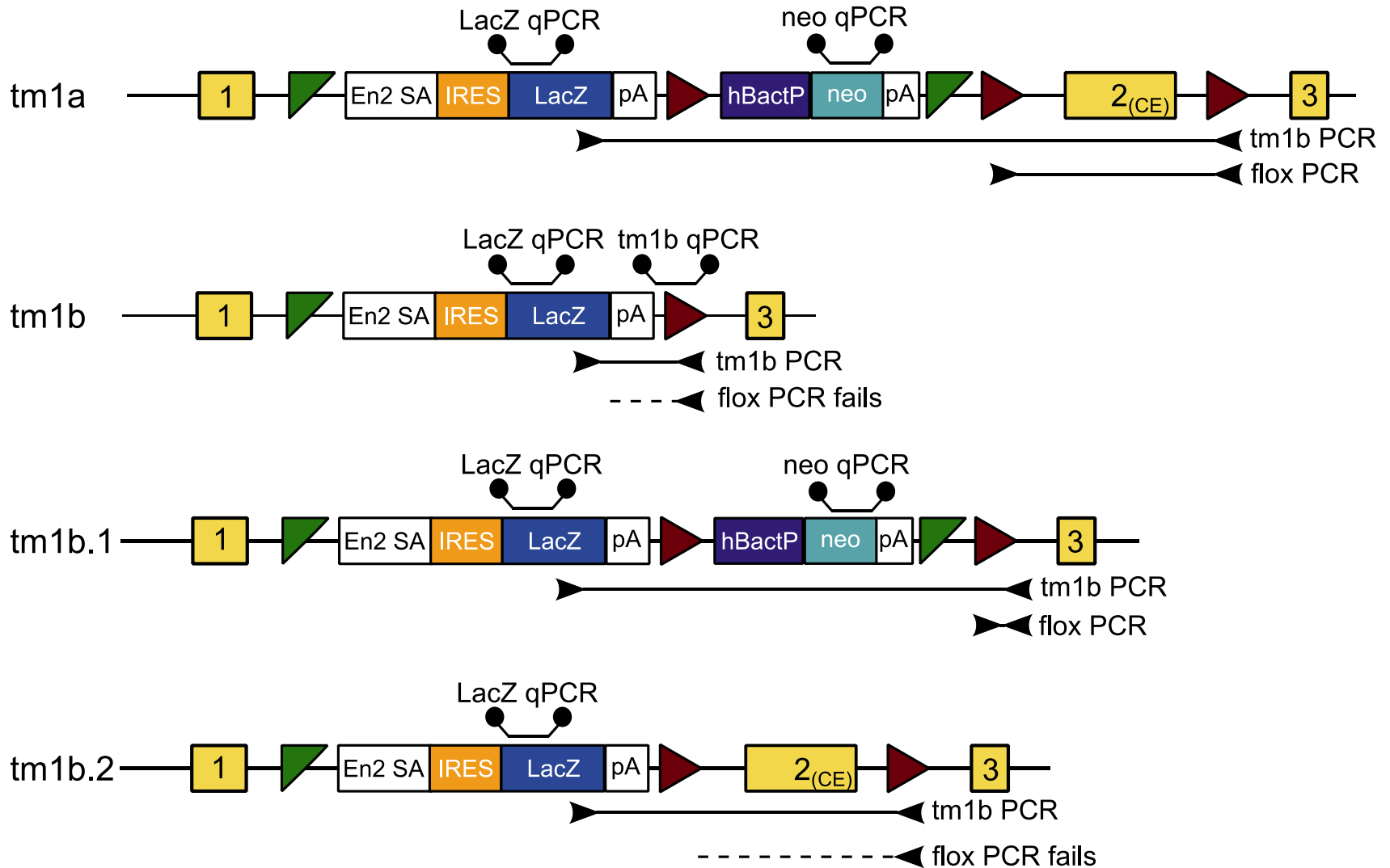
Cre breeding



- Traditional breeding takes a minimum of two generations to convert $tm1a$ to $tm1b$ with the Cre driver removed
- Use of a cell permeable Cre or a Cre driver with maternal expression can reduce this by a generation

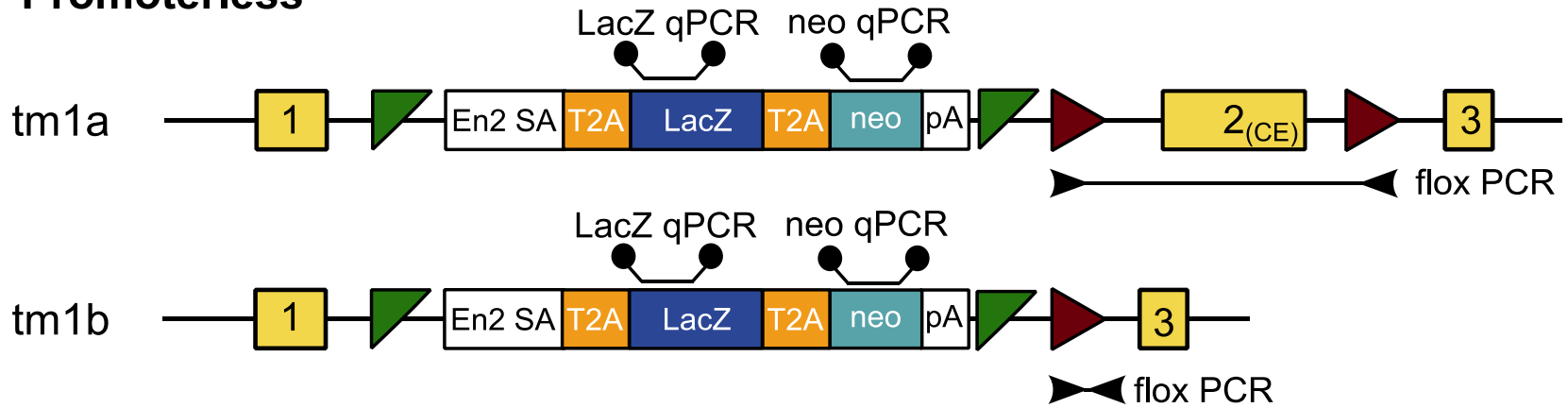
Tm1b conversion detection: promoter-driven lines

Promoter-driven



Tm1b conversion detection: promoterless lines

Promoterless



Tm1b conversion screen outcomes

Promoter-driven lines

Conversion	srPCR assay		qPCR assay		
	tm1b	Flox	tm1b_prom	LacZ count	Neo count
no conversion (tm1a)	2337bp + CE size	dependent on CE size	fail	no change	no change
tm1b.1 - only critical exon (CE) removed	2291bp	128bp	fail	no change	no change
tm1b.2 - only <i>neo</i> removed	426bp + CE size	fail	fail	no change	-1 copy
Full conversion to tm1b	380bp	fail	pass	no change	-1 copy

Promoterless lines

Conversion	srPCR assay		qPCR assay		
	tm1b	Flox	tm1b_prom	LacZ count	Neo count
no conversion (tm1a)	Fail	dependent on CE size	fail	no change	no change
tm1b	Fail	128bp	fail	no change	no change

- A combination of six PCR-based assays can be used to detect the Cre driver, the mouse genotype and any conversion from the tm1a to the tm1b forms of the mutant allele.
- Using this method, the mice can be genotyped in a mutant strain-independent manner using the LacZ qPCR assay which counts the number of copies of the cassette present and is unaffected by tm1b conversion.
- If no qPCR technologies are available then the short range PCR (srPCR) can be used to detect conversion and the mice genotyped by other methods (eg gene specific PCR assays designed to discriminate between the mutant and WT alleles).

Primer sequences for short-range endpoint PCR

Short range primer sequences

Primer name	Sequence
Cre_F	CATTTGGGCCAGCTAAACAT
Cre_R	TAAGCAATCCCCAGAAATGC
Floxed PNF	ATCCGGGGGTACCGCGTCGAG
Floxed LR	ACTGATGGCGAGCTCAGACC
Tm1b_prom_F	CGGTCGCTACCATTACCAGT

PCR assays for tm1b and Cre detection

Assay	F primer	R primer	Size (bp)
Cre	Cre_F	Cre_R	233
Flox	Floxed PNF	Floxed LR	variable
Tm1b	Tm1b_prom_F	Floxed LR	380

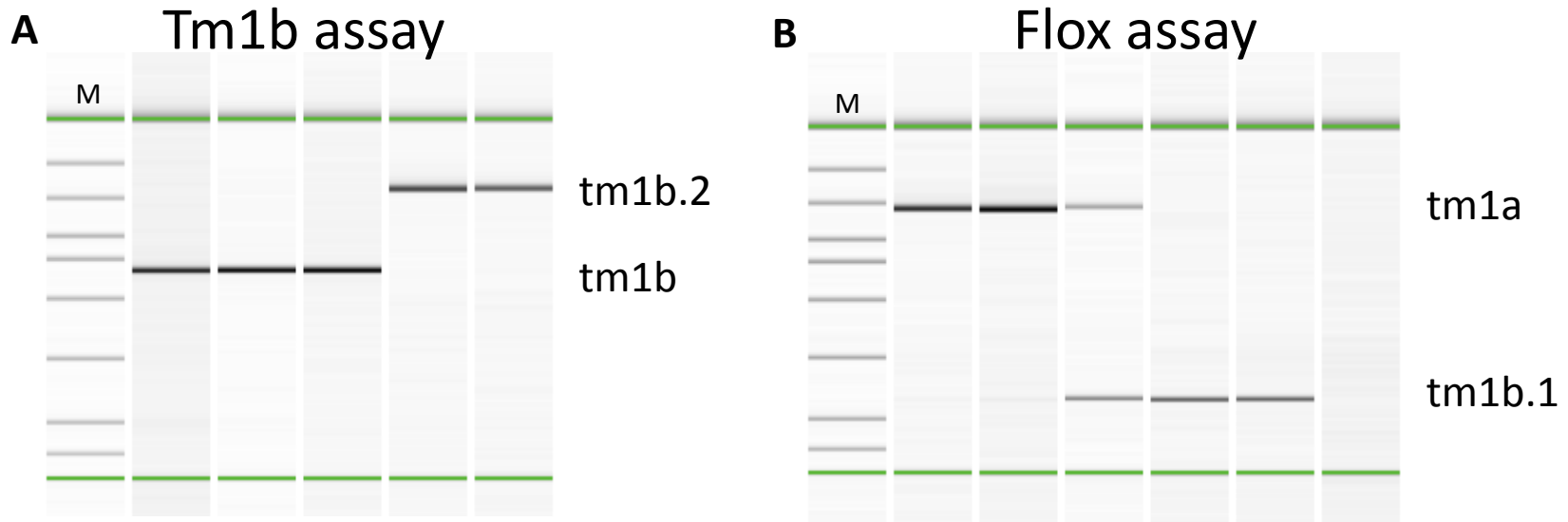
PCR reagents and conditions

Reagent	volume (ul)
Primer 1 (10uM)	0.3
Primer 2 (10uM)	0.3
MgCl ₂ (50mM)	0.45
10x Buffer	1.5
dNTP (100mM)	0.15
Taq*	0.15
ddH ₂ O	11.15
DNA	1
Total	15

- 1 94 °C 5 min
- 2 94 °C 30 sec
- 3 58 °C 30 sec
- 4 72 °C 90 sec
- 5 Go to '2' + 34 cycles
- 6 72 °C 5 min
- 7 12 °C forever

*Platinum Taq is used in the PCR reaction (Invitrogen)

Short range end-point PCR assays



- Size differences in the end-point PCR reactions can detect partial and mosaic conversion of the mutant allele (data shown for promoter-driven lines).
- **(A)** The tm1b assay detects full conversion to tm1b in three samples and partial conversion to tm1b.2 in two others.
- **(B)** The flox assay (performed on different samples and shown here for illustrative purposes) detects conversion to tm1b.1 in two samples and no conversion in two others. The remaining sample shows mosaicism where both the tm1a and tmb.1 forms are detected.

qPCR assays

Real-time qPCR assay sequences

Assay	F primer	R primer	Probe
LacZ	GGAGTGCGATCTTCTGAGG	CGCATCGTAACCGTGCATC	CGATACTGTCGTCGTCCCCTCAAACCTG
Neo	GGTGGAGAGGCTATTCCGGC	GAACACGGCGGCATCAG	TGGGCACAACAGACAATCGGCTG
Tm1b_prom	GTCCAAACTCATCAATGTATCTTATCATGT	GATGGCGAGCTCAGACCATAA	TGGATCCGGAATAAAGTTCGTA
Cre	ACGTACTGACGGTGGGAGAA	GTGCTAACCAGCGTTTTTCGTT	CTGCCAATATGGATTAACA

qPCR reagents and conditions

Reagent	Volume (ul)
2x GTXpress buffer*	5
60x TaqMan assay	0.166
ddH ₂ O	3.334
20x endogenous probe (Tfrc)*	0.5
DNA	1
Total	10

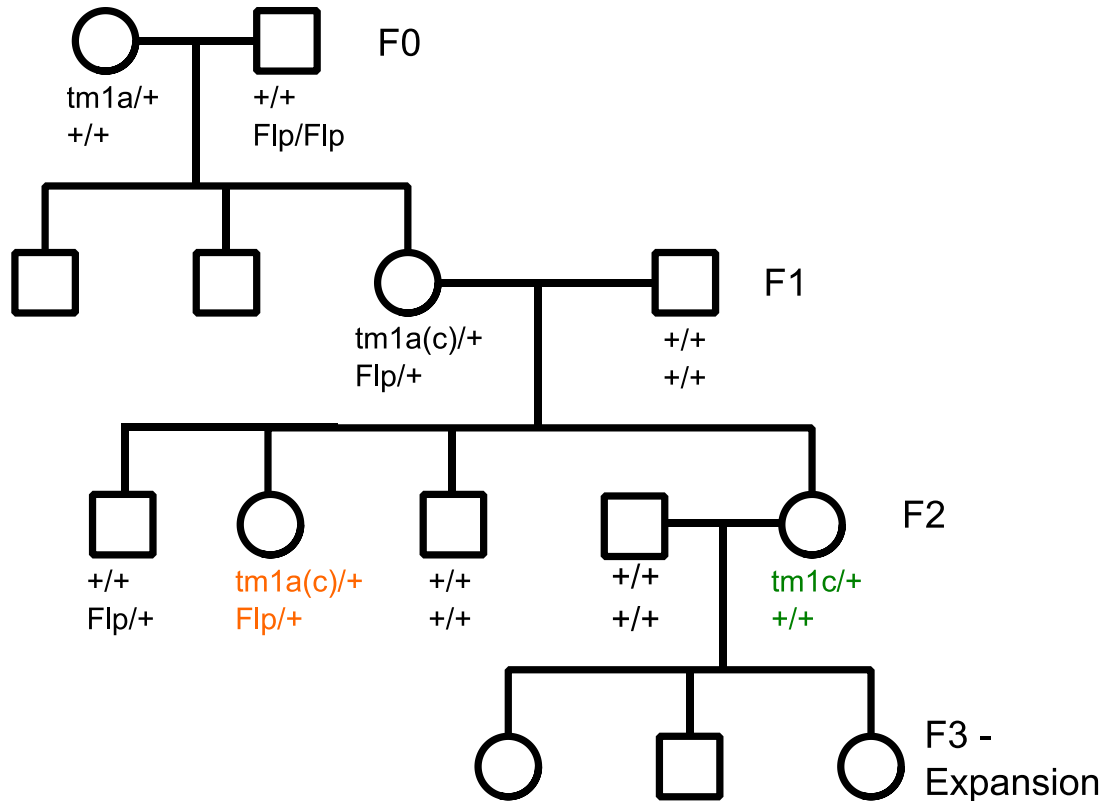
95°C 20sec } x1
 95°C 10sec } x35
 60°C 30sec }

* Life Technologies

Real-time qPCR assays are analysed using the $2^{-\Delta\Delta Ct}$ method using known homozygotes as calibration controls

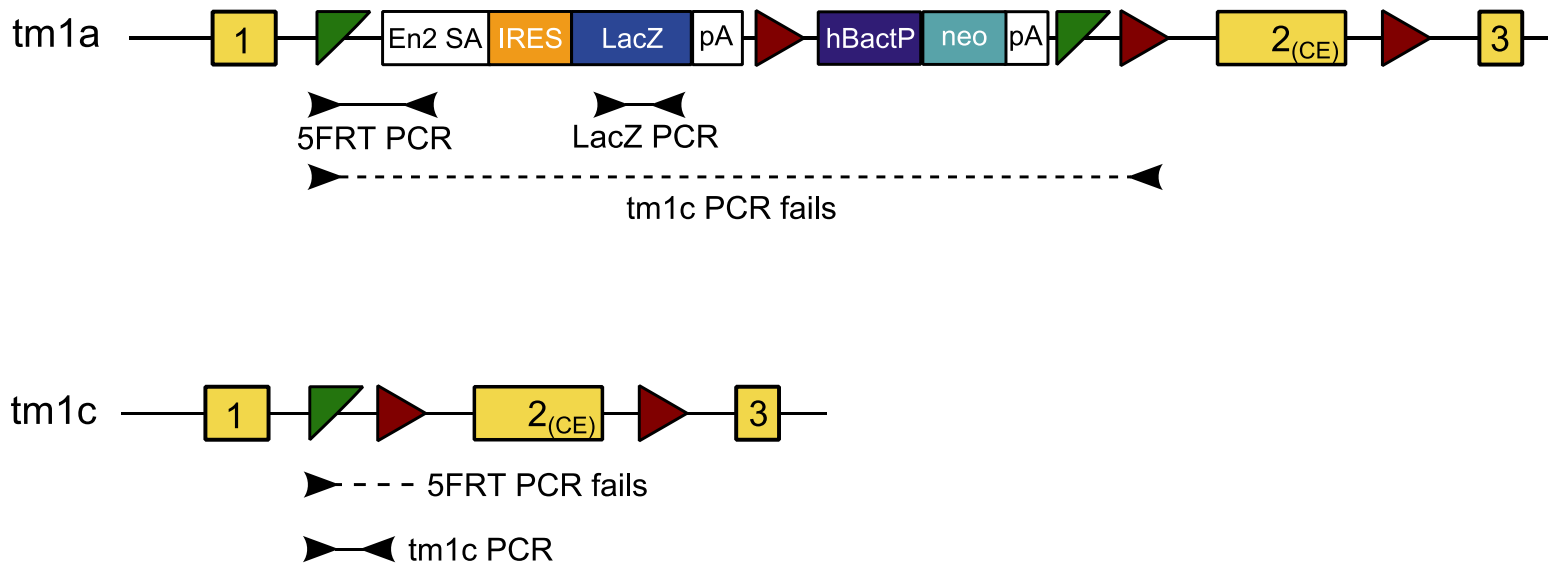
TM1C CONVERSION

Flp breeding



- Traditional breeding takes a minimum of two generations to convert *tm1a* to *tm1c* with the Flp driver removed
- Use a Flp driver with maternal expression can reduce this by a generation

Tm1c conversion detection



Tm1c conversion screen outcomes

	LacZ assay	Tm1c assay	5'FRT assay
Cassette removed (tm1c)	fail	pass	fail
Cassette not removed (tm1a)	pass	fail	pass

- If using the 3-assay short-range genotyping system provided by the Sanger Institute Mouse Genetics Project (MGP) please note that the conversion to tm1c will result in the WT-specific assay amplifying a product in most cases.
 - The size of this product will be the native WT size + 218bp (linker sequence) – the sequence deleted during the targeted mutation design

Genotyping and conversion detection

Example gel image of genotyping and tm1c conversion detection using MGP strategy

Mouse	SrPCR					qPCR	
	WT PCR	Mut PCR	LacZ PCR	5'FRT	Tm1c	FLPeR	
MMFP12.1a						MMFP12.1a	+/+ 0
MMFP12.1b						MMFP12.1b	+/+ 0
MMFP12.1c						MMFP12.1c	+/+ 0
MMFP12.1d						MMFP12.1d	+/- 0.479
MMFP12.1e						MMFP12.1e	+/- 0.481
MMFP12.1f						MMFP12.1f	+/- 0.596
MMFP12.1g						MMFP12.1g	+/- 0.479
MMFP12.1h						MMFP12.1h	+/- 0.455
MMFP12.1i						MMFP12.1i	+/- 0.524
+ve							

- Conversion is observed in 6 mice and 3 additional mice are WT
- Two WT bands are amplified in converted heterozygotes due to linker sequence remaining in the tm1c allele
- Mouse MMFP12.1f shows mosaic conversion and amplifies products in both 5FRT and tm1c assays

Primer sequences for short-range endpoint PCR

Short range primer sequences

Primer name	Sequence
5FRT_F	AGGCGCATAACGATACCACGAT
5FRT_R	CCACAACGGGTTCTTCTGTT
tm1c_F	AAGGCGCATAACGATACCAC
tm1c_R	CCGCCTACTGCGACTATAGAGA
LacZ_F	ATCACGACGCGCTGTATC
LacZ_R	ACATCGGGCAAATAATATCG
FlpE_F	CCTAAGGTCCTGGTTCGTCA
FlpE_R	TTGTTGCTTTTTGCGTCTTG
FlpO_F	CCACAGCAAGAAGATGCTGA
FlpO_R	TCTTGATGTCGCTGAACCTG

PCR reagents and conditions

Reagent	volume (ul)
Primer 1 (10uM)	0.3
Primer 2 (10uM)	0.3
MgCl ₂ (50mM)	0.45
10x Buffer	1.5
dNTP (100mM)	0.15
Taq*	0.15
ddH ₂ O	11.15
DNA	1
Total	15

- 1 94 °C 5 min
- 2 94 °C 30 sec
- 3 58 °C 30 sec
- 4 72 °C 45 sec
- 5 Go to '2' + 34 cycles
- 6 72 °C 5 min
- 7 12 °C forever

*Platinum Taq is used in the PCR reaction (Invitrogen)

PCR assays for tm1c and Flp detection

Assay	F primer	R primer	Size (bp)
5' FRT	5FRT_F	5FRT_R	204
Tm1c	tm1c_F	tm1c_R	218
LacZ	LacZ_F	LacZ_R	108
FlpE	FlpE_F	FlpE_R	229
FlpO	FlpO_F	FlpO_R	176

qPCR assays

Real-time qPCR assay sequences

Assay	F primer	R primer	Probe
LacZ	GGAGTGCATCTTCCTGAGG	CGCATCGTAACCGTGCATC	CGATACTGTCGTCGTCCCCTCAAACCTG
FlpE	CATCTGGGAGATCACTGAGAAAATACT	TGAAAGTAGCTAGGAAGAGGAATTGGT	AAGTTTTTGTTTTGTAAATCTC
FlpO	GCAGGTTACCAAGACCAAGA	CTGCCGCAGTTGATGAATGTG	CCTGTACCAGTTCCTGTTCC

qPCR reagents and conditions

Reagent	Volume (ul)
2x GTXpress buffer*	5
60x TaqMan assay	0.166
ddH ₂ O	3.334
20x endogenous probe (Tfrc)*	0.5
DNA	1
Total	10

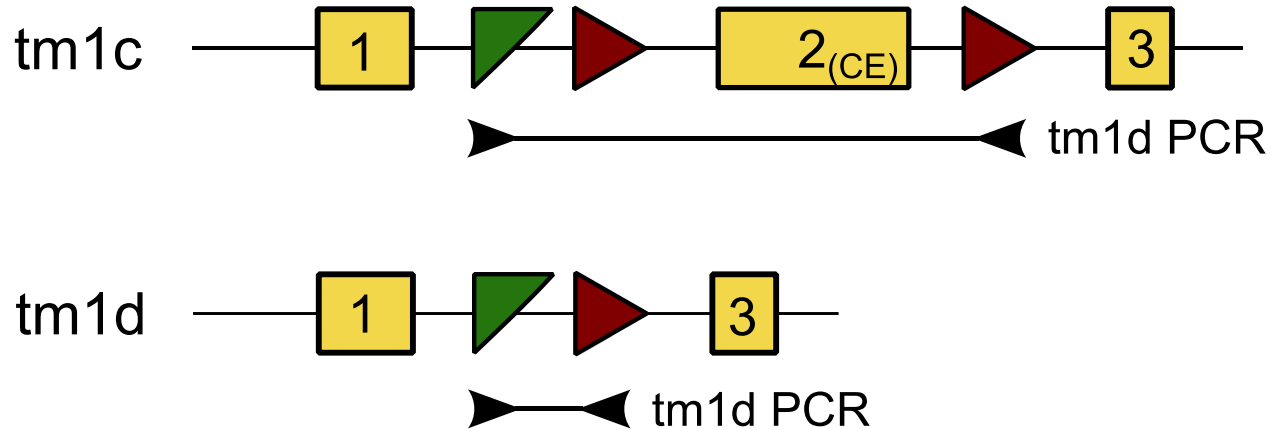
95°C 20sec } x1
95°C 10sec } x35
60°C 30sec }

* Life Technologies

Real-time qPCR assays are analysed using the $2^{-\Delta\Delta Ct}$ method using known homozygotes as calibration controls

TM1D CONVERSION

Tm1d conversion detection



- If recombination has occurred this product size will be fixed at 174bp
- If Cre mediated recombination has not occurred the PCR product size will be 155bp plus the size of floxed critical exon region

Genotyping and conversion detection

Example gel image of genotyping and tm1c conversion detection using MGP strategy

	WT PCR	Mut PCR	LacZ PCR	Tm1c PCR	Tm1d PCR	Genotype
MMAH9.1a						+/+
MMAH9.1b						-/-
MMAH9.1c						-/-
MMAH9.1d						-/-
MMAH9.1e						+/-
MMAH9.1f						-/-

- Conversion is observed in five mice and one mouse is WT
- The tm1c assay and lacZ assays fail by design
- The WT assay fails on the tm1d allele as the reverse primer is designed in the (now deleted) floxed region.

Primer sequences for short-range endpoint PCR

Short range primer sequences

Primer name	Sequence
tm1c_F	AAGGCGCATAACGATACCAC
Floxed LR	ACTGATGGCGAGCTCAGACC

PCR assays for tm1d detection

Assay	F primer	R primer	Size (bp)
Tm1d	Tm1c_F	Floxed LR	174

PCR reagents and conditions

Reagent	volume (ul)	
Primer 1 (10uM)	0.3	1 94 °C 5 min
Primer 2 (10uM)	0.3	2 94 °C 30 sec
MgCl ₂ (50mM)	0.45	3 58 °C 30 sec
10x Buffer	1.5	4 72 °C 45 sec
dNTP (100mM)	0.15	5 Go to '2' + 34 cycles
Taq*	0.15	6 72 °C 5 min
ddH ₂ O	11.15	7 12 °C forever
DNA	1	
Total	15	

*Platinum Taq is used in the PCR reaction (Invitrogen)

References

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