tm1a allele conversion

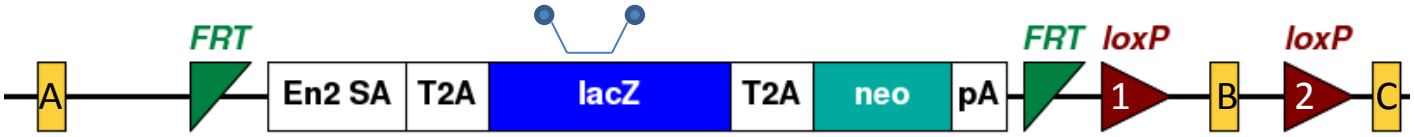
Mouse Genetics Project
Wellcome Trust Sanger Institute

Conversion to tm1b after crossing with a source of Cre

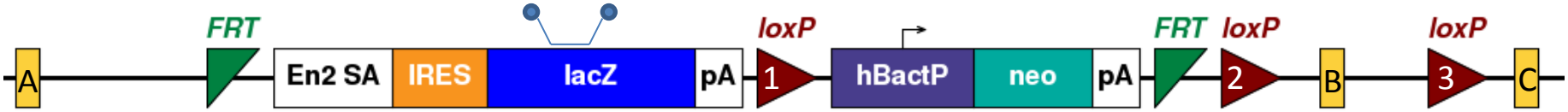
Selection cassettes

- There are two main cassette designs used in the high throughput gene targeting labs
- A LacZ qPCR assay can be used as a high-throughput method of determining the presence of the mutant gene, but it will not discriminate between converted forms of the b allele

Promoterless selection cassette – tm1a configuration



Promoter-driven selection cassette - – tm1a configuration



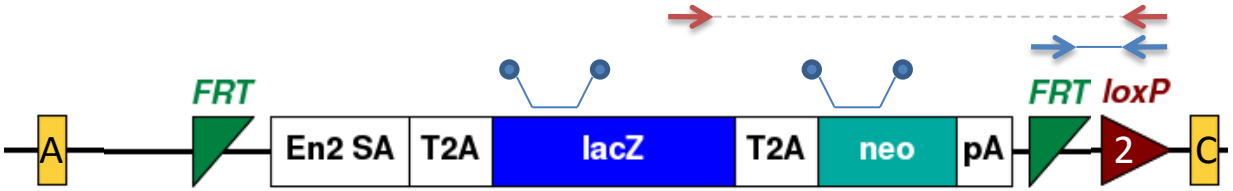

qPCR assay

Promoterless – cre recombination

Outcome 1: no recombination event occurs - tm1a allele

- LoxP PCR size same as tm1a
- Neo count unaltered
- Tm1b PCR ~2.5kb (or fails)

Outcome 1: recombination occurs - tm1b allele



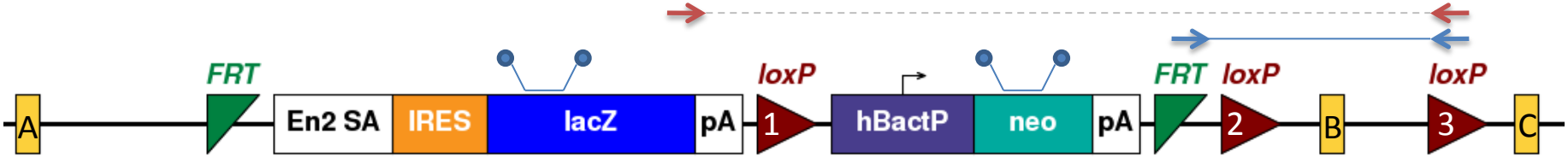
- LoxP PCR size reduced to ~130bp
- Neo count unaltered
- tm1b PCR ~1.5kb (or fails)

→ **LoxP PCR**

→ **tm1b PCR**

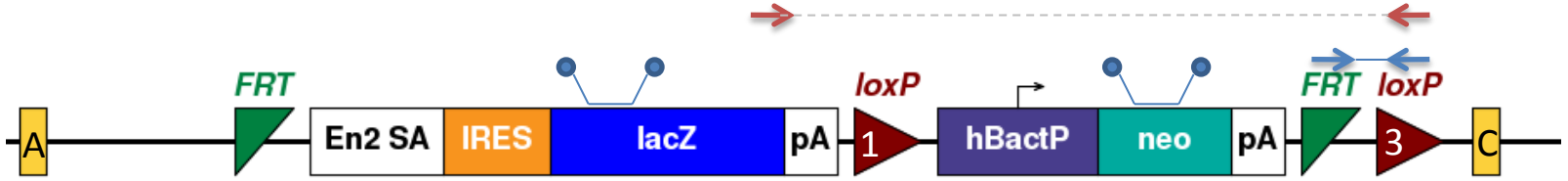
Promoter-driven cre recombination

Outcome 1: no recombination event occurs – tm1a allele



- LoxP PCR size same as tm1a
- Neo count unaltered
- Tm1b PCR fails

Outcome 2: only critical exon removed – tm1b.1 allele



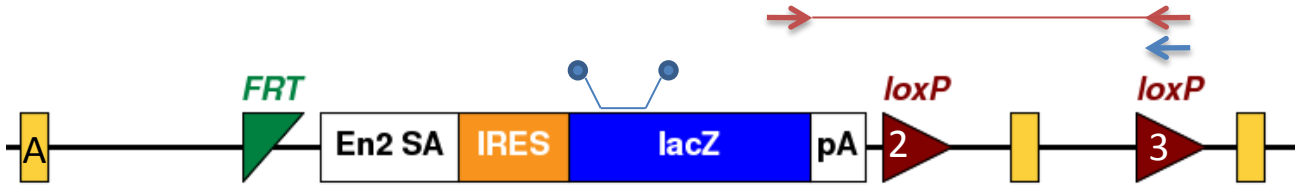
- LoxP PCR size reduced to ~130bp
- Neo count unaltered
- tm1b PCR fails
- Configuration not stable if Cre is still present

→ **LoxP PCR**

→ **tm1b PCR**

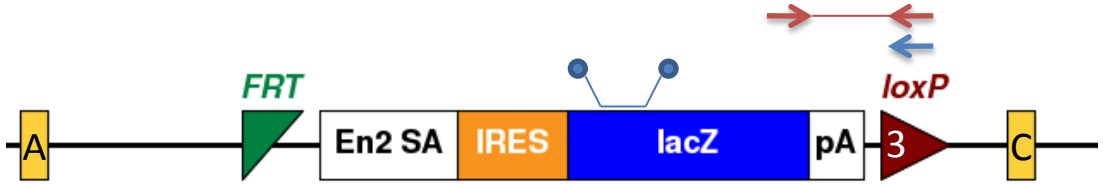
Promoter-driven cre recombination II

Outcome 3: only Neo marker removed – tm1b.2 allele



- LoxP PCR fails
- Neo count reduced by 1 copy
- tm1b PCR gives a ~1kb product

Outcome 4: critical exon and neo marker removed – tm1b allele

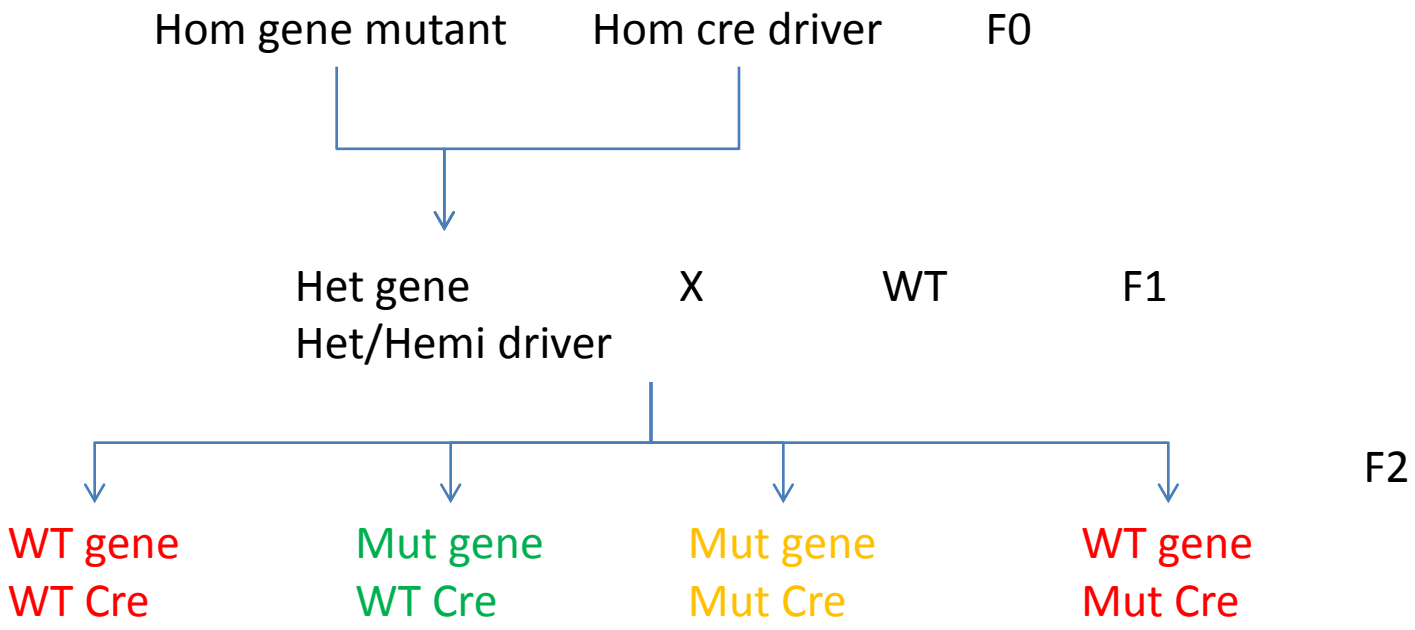


- LoxP PCR fails
- Neo count reduced by 1 copy
- tm1b PCR works

→ **LoxP PCR**

→ **tm1b PCR**

Mouse breeding



- Mut gene / WT Cre with full conversion to Tm1b is most desirable
  - The line is expected to be stable at this point and may be genotyped with just the normal gene-specific srPCR primers (although not Neo count qPCR if the line is promoter-driven)

Genotyping and detecting conversion

- Gene and Cre detectors:
  - Gene WT-specific srPCR
  - Gene mutant-specific srPCR
  - Cre-specific srPCR (or qPCR)
  - Neo count qPCR (cannot distinguish between gene and Cre versions if Neo is present in the Cre driver cassette)
  - LacZ count qPCR
- Recombination detection:
  - Neo count qPCR
  - LoxP PCR
  - Tm1b PCR

- *While Neo count qPCR gives extra information it is not critical for correct genotype confirmation*

F2 genotyping outcomes

Promoter- driven lines for further breeding

Gene WT	Gene Mut	Cre	Lox P	Neo Count	Tm1b	Mutant gene present?	Cre driver present?	Tm1a conversion
pass	pass	pass	tm1a size	2	fail	yes	yes	no
pass	pass	pass	200bp	2	fail	yes	yes	Tm1b.1
pass	pass	pass	fail	1	pass	yes	yes	Tm1b
pass	pass	fail	tm1a size	1	fail	yes	no	no
pass	pass	fail	200bp	1	fail	yes	no	Tm1b.1
pass	pass	fail	fail	0	pass	yes	no	Tm1b
pass	pass	pass	fail	1	large	yes	yes	Tm1b.2
pass	pass	fail	fail	0	large	yes	no	Tm1b.2

	breed with WT to produce a stable colony
	breed with WT and selectively remove Cre progeny from next generation
	do not breed or breed with a Cre driver mouse to attempt another allele conversion
	genotype next generation - conversion in germ line may have occured

Promoterless lines for further breeding

Gene WT	Gene Mut	Cre	Lox P	Neo Count	Tm1b	Mutant gene present?	Cre driver present?	Tm1a conversion
pass	pass	pass	tm1a size	2	fail	yes	yes	no
pass	pass	pass	200bp	2	fail	yes	yes	Tm1b
pass	pass	fail	tm1a size	1	fail	yes	no	no
pass	pass	fail	200bp	1	fail	yes	no	Tm1b

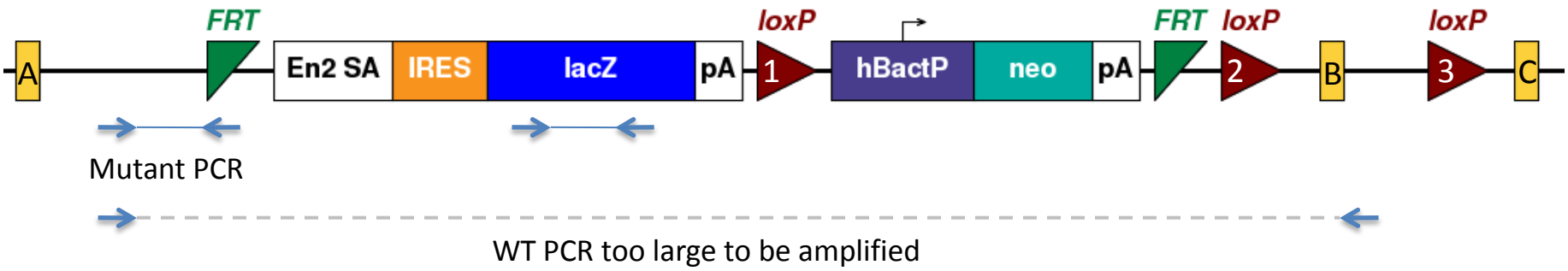
	breed with WT to produce a stable colony
	breed with WT and selectively remove Cre progeny from next generation
	do not breed or breed with a Cre driver mouse to attempt another allele conversion
	genotype next generation - conversion in germ line may have occured

- Gene mutant-specific PCR fail results not shown.
- Please note that Neo count in this example assumes the presence of Neo in the Cre driver cassette. Reduce expected Neo count by one if this is not the case

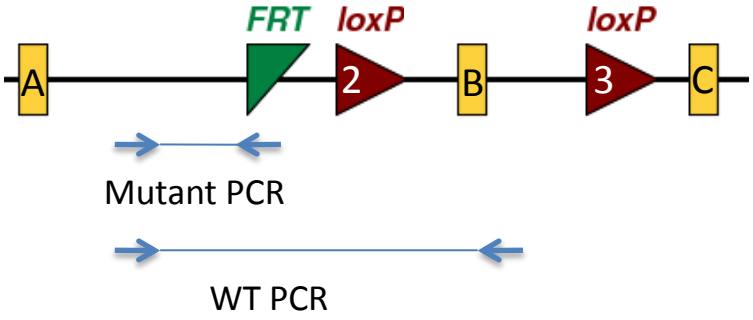
Conversion to tm1c after crossing with a source of Flp

Genotyping flipped out knockout-first alleles

Knockout-first mutant allele (tm1a) (cassette not to scale)



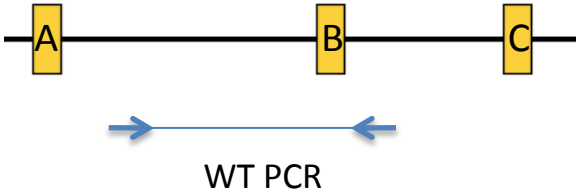
Flipped out mutant allele (tm1c) – WT function restored. One FRT site remains



LacZ PCR fails as cassette is missing

WT PCR works but is a different size to the native WT (actual size difference is allele specific)

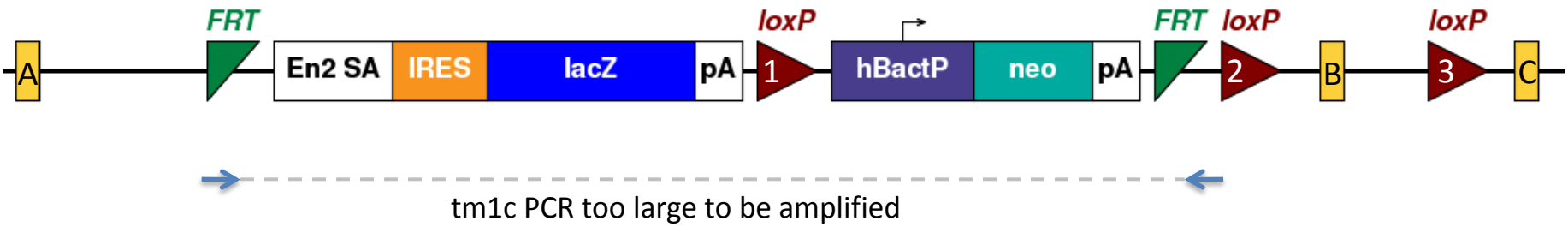
Native WT allele

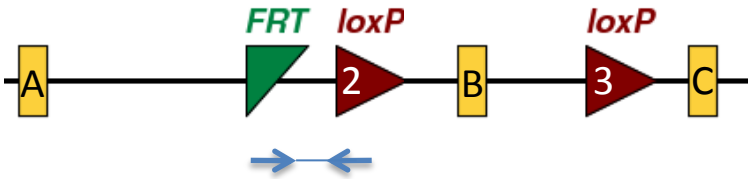


LacZ PCR fails as cassette is missing

Mutant PCR fails as cassette is missing

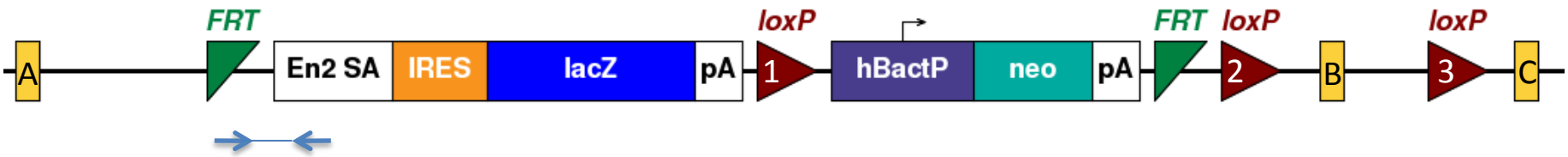
tm1c PCR



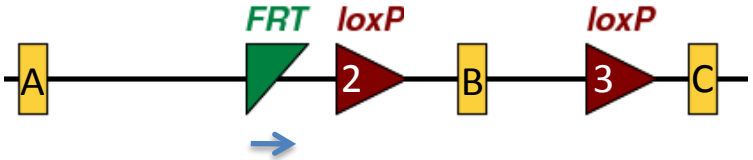


- Presence of band calls the Tm1c allele

5’FRT PCR



5’FRT PCR works



PCR fails due to missing primer binding site

- Absence of band in and presence of a mutant-specific band calls the tm1c allele
- The 5’FRT PCR can also be used as a 5’ element integrity check in tm1a alleles

Expected results and assay conditions

	WT assay	Mut assay	LacZ assay	Tm1c assay	5'FRT assay
Cassette removed (tm1c)	pass (size different to native WT)	pass	fail	pass	fail
Cassette not removed (tm1a)	fail	pass	pass	fail	pass

Assay	Forward primer	Reverse primer	Size (bp)	PCR
5' FRT	5FRT_F	5FRT_R	204	TA58
Tm1c	tm1c_F	tm1c_R	218	TA58
LacZ	LacZ_2_small_F	LacZ_2_small_R	108	TA58

Primer name	Sequence
5FRT_F	AGGCGCATAACGATACCACGAT
5FRT_R	CCACAACGGGTTCTTCTGTT
tm1c_F	AAGGCGCATAACGATACCAC
tm1c_R	CCGCCTACTGCGACTATAGAGA
LacZ_2_small_F	ATCACGACGCGCTGTATC
LacZ_2_small_R	ACATCGGGCAAATAATATCG

Reagent	volume (ul)
Primer 1 (10uM)	0.4
Primer 2 (10uM)	0.4
MgCl2 (50mM)	0.6
10x Buffer	2
dNTP (100mM)	0.2
PtTaq*	0.2
H20	15.2
DNA	1
Total	**20**

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

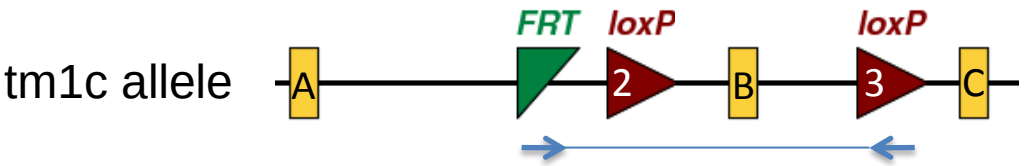
TA58

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

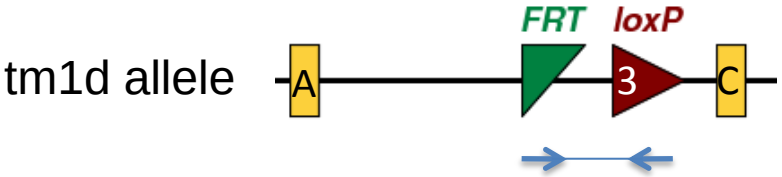
Conversion to tm1d after crossing a tm1c mouse with a source of Cre

Genotyping tm1d alleles

• For lines that have previously undergone cassette excision to produce the tm1c allele, a different assay is needed.



PCR size 155bp+ critical exon region



PCR size 174bp

• If Cre mediated recombination has not occurred the PCR product size will be 155bp plus the size of critical exon region (usually ~900bp to 1.5kb)
• If recombination has occurred this product size will be fixed at 174bp
• If the critical exon region is very large then a gene-specific reverse primer can be designed within it; amplification of a band would strongly suggest that the Tm1d allele has not been produced
• LacZ qPCR assay will fail when the tm1c or tm1d form is present

PCR primers and conditions

Assay	F primer	R primer	Size (bp)	Type	PCR
Cre	Cre_F	Cre_R	233	Cre	TA58
LoxP	Floxed PNF	Floxed LR	800 - 1kb	LoxP	TA58
Tm1b	Tm1b_prom_F	Floxed LR	380	cassette	TA58
Tm1d	Tm1c_F	Floxed LR	174	cassette	TA58

Primer name	Sequence
Cre_F	CATTTGGGCCAGCTAAACAT
Cre_R	TAAGCAATCCCCAGAAATGC
CAS_R1_Term	TCGTGGTATCGTTATGCGCC
Floxed PNF	ATCCGGGGGTACCGCGTCGAG
Floxed LR	ACTGATGGCGAGCTCAGACC
Tm1b_prom_F	CGGTCGCTACCATTACCAGT
Tm1c_F	AAGGCGCATAACGATACCAC

Reagent	volume (ul)
Primer 1 (10uM)	0.4
Primer 2 (10uM)	0.4
MgCl2 (50mM)	0.6
10x Buffer	2
dNTP (100mM)	0.2
PtTaq*	0.2
H20	15.2
DNA	1
Total	**20**

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

TA58

1	94 ^{o}C	5 min
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3	58 ^{o}C	30 sec
4	72 ^{o}C	45 sec
5	Go to '2' + 34 cycles	
6	72 ^{o}C	5 min
7	12 ^{o}C	forever