GENSAT PCR Design Guideline

1. Log onto http://www.gensat.org/bacreport.jsp website. Locate the gene of interest then find the A-homology arm (A-box) 3' Primer for that gene.
2. Open another window or tab on your web browser and log onto http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=10090. In the search line type the name of the gene of interest and search the mapviewer by clicking on the Go button.
3. Under map element select a match that most closely fits your gene (this may need to be repeated if you do not see your gene on the chromosome map on the next page). If the gene is displayed, under maps and options click Download/View Sequence/Evidence. On the next page click display and highlight **only** the DNA sequence.
4. Open Vector NTI (or other sequence editor program), under **file**—click **Create new sequence / Using Sequence Editor (DNA/RNA)**…
5. Name the new DNA/RNA molecule then click on the **Sequences and Maps** tab and click **edit sequence**. In the new window click **paste** to paste the DNA sequence.
6. Using **Find Sequence** tab, enter the appropriate A-homology arm (A-box) 3' Primer (found in the BAC Report page) and search using both direct and complement. If it does not find the sequence, then reverse the sequence and search for that again by both direct and complement.
7. Mark the A-box primer sequence location by highlighting it or underlining it.
8. Locate the region of DNA transcriptionally upstream of the now marked 3' A-box primer and highlight it.
9. Design a transcriptional sense gene specific forward primer (GSP) (this will be opposite strand of the 3' A-box primer).
10. Analyze GSP candidates to ensure the Tm is between 55-60 and the GC content 40-60%. Pick primers low in secondary structure and with 3'ends (ΔG ~ -9kCal/mol) with 3 A/T and 2 G/C in last 5 nucleotides.
11. Use this new forward primer you just designed with one of the given eGFP reverse primer sequences (CAGGGCACGGGCAGCTT, TAGCGGCTGAAGCACTGCA, GGTCGGGGTAGCGGCTGAA, or CTTCGGGCATGGCGGACTT).
12. Use the attached GENSAT Stringent Protocol first. Should that protocol not work, try the GENSAT Non Stringent Protocol if needed.
13. Amplicon should yield >300 bp fragment on agarose gel (depending on placement of forward primer).
14. Optimize by: 1). Decreasing $MgCl_2$ if nonspecific banding is prominent 2). Use final concentration of 1.3M Betaine and 1.3% DMSO in PCR cocktail if reaction is weak or fails.

Procedure developed by Brandon Willis, Manager
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GENOTYPING BY PCR PROTOCOL

MUTANT MOUSE REGIONAL RESOURCE CENTER: UC DAVIS
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530-754-MMRRC

NAME OF PCR: **GENSAT Stringent PCR Protocol**

Protocol:

Reagent/ Constituent	Volume (µL)
Water	11.275
10x Buffer	2.5
$MgCl_2$ (stock concentration is 25mM)	1.7
Betaine (stock concentration is 5M)	6.5
dNTPs (stock concentration is 10mM)	0.5
DMSO	0.325
Primer 1 (stock concentration is 20µM) Gene Specific Fwd Primer (GSP)	0.5
Primer 2 (stock concentration is 20µM) GFP R2	0.5
Taq Polymerase (5Units/µL)	0.2
DNA extracted with ☐ NaOH ☒ Proteinase K ☐ Other:	1.0
TOTAL VOLUME OF REACTION:	**25µL**

Comments on protocol:

- Use Touch-Down cycling protocol-first 10 cycles anneal at 65° C decreasing in temperature by 1.0 ° C; next 30 cycles anneal at 55° C.
- Betaine/DMSO is standardized due to high GC content in promoter regions. Also, may adjust $MgCl_2$ to increase reaction or decrease non specific amplifications.

Strategy:

Steps		Temp (°C)	Time (m:ss)	# of Cycles
1. Initiation/Melting	HOT START? ☐	94	5:00	**1**
2. Denaturation		94	0:15	
3. Annealing	steps 2-3-4 will cycle in sequence	65 to 55 (↓1°C/cycle)	0:30	**40x**
4. Elongation		72	0:40	
5. Amplification		72	5:00	**1**
6. Finish		4	Hold	n/a

Primers:

Name	Nucleotide Sequence (5' - 3')
1: Gene Specific Fwd Primer (GSP)	
2: GFP R2	TAGCGGCTGAAGCACTGCA

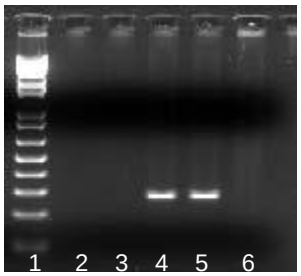
Electrophoresis Protocol:

Agarose: 1.5% **V:** 90

Estimated Running Time: 90 **min.**

Band	Genotype
none	Wild-type
~300 bp	Transgene +

Example Gel Photo



Lanes
1: 1 kb+ ladder
(Invitrogen, Cat. #10787-026)
2: water control
3: wild-type control
4 & 5: Emx1 tg/+
6: other GENSAT line

GENOTYPING BY PCR PROTOCOL

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NAME OF PCR: GENSAT Non-Stringent PCR Protocol

Protocol:

Reagent/ Constituent	Volume (µL)
Water	11.275
10x Buffer	2.5
$MgCl_2$ (stock concentration is 25mM)	1.7
Betaine (stock concentration is 5M)	6.5
dNTPs (stock concentration is 10mM)	0.5
DMSO	0.325
Primer 1 (stock concentration is 20µM) Gene Specific Fwd Primer (GSP)	0.5
Primer 2 (stock concentration is 20µM) GFP R2	0.5
Taq Polymerase (5Units/µL)	0.2
DNA extracted with ☐ NaOH ☒ Proteinase K ☐ Other:	1.0
TOTAL VOLUME OF REACTION:	**25µL**

Comments on protocol:

- Use Touch-Down cycling protocol-Anneal at 60° C for 5 cycles, next 5 cycles anneal at 58° C, next 10 cycles anneal at 55° C, and final 10 cycles anneal at 50 ° C.
- Betaine/DMSO is standardized due to high GC content in promoter regions. Also, may adjust $MgCl_2$ to increase reaction or decrease non specific amplifications.

Strategy:

Steps		Temp (°C)	Time (m:ss)	# of Cycles
1. Initiation/Melting	HOT START? ☐	95	5:00	**1**
2. Denaturation		95	0:20	
3. Annealing	steps 2-3-4 will cycle in sequence	60 to 50	0:30	**35x**
4. Elongation		72	0:40	
5. Amplification		72	5:00	**1**
6. Finish		4	Hold	n/a

Primers:

Name	Nucleotide Sequence (5' - 3')
1: Gene Specific Fwd Primer (GSP)	
2: GFP R2	TAGCGGCTGAAGCACTGCA

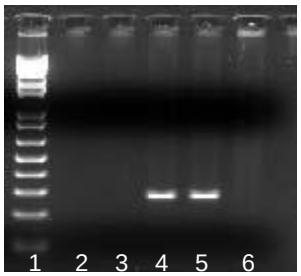
Electrophoresis Protocol:

Agarose: 1.5% **V:** 90

Estimated Running Time: 90 **min.**

Band	Genotype
none	Wild-type
~300 bp	Transgene +

Example Gel Photo



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