

INTEGRATION SITE PCR: PCR STRATEGY TO CONFIRM THE GENOMIC INTEGRATIONSITE OF GENE TRAP CASSETTES

An alternative to the inverse PCR to confirm the correct genomic integration site of any ES cell clone is to use the sequence which we initially retrieved by iPCR to map the exact genomic location of the mutagen. By retrieving the genomic sequence of a particular gene from e.g. MGI, you can easily design primers flanking the "mapped sequence" and integration site.

Our automatically designed clone-specific-primers flanking the locus of integration are noted for each clone on the QC sheet. Combining these with the gene trap specific primers, six PCR reactions are run with different primer combinations on the genomic DNA extracted from wildtype ES cells (AN3-12) and Haplobank ES cell clones. Based on the size of the amplicons generated by the individual PCR reactions, you can confirm the integration of the gene trap in its correct location as well as assess the status of homo- or heterozygosity of integration.

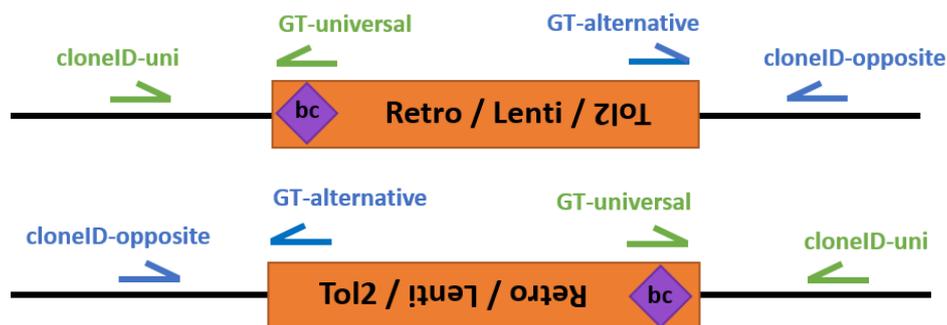
There are five different gene trap internal primers: The **GT-universal** primer is the same in all mutagens because it is located at the 5' or 3' end of the gene trap which contains the barcode (Retro/Lenti: 5'end; Tol2pA/Tol2GT: 3'end). The second gene trap internal primer (**GT-alternative**) is individual for every mutagen (Retro/Lenti/Tol2pA/Tol2GT) and is located at the 5' or 3' end of the gene trap (Retro/Lenti: 3'end; Tol2pA/Tol2GT: 5'end).

Based of the inserted mutagen and the strand of integration the clone-specific primers are named **cloneID-uni** and **cloneID-opposite**.

Per clone following three different primer combinations are set up:

- cloneID-uni + GT-universal
- cloneID-opposite + GT-alternative (GT = Tol2pA, Tol2GT, Retro or Lenti; dependent on clone mutagen)
- cloneID-uni + cloneID-opposite

Each combination will be used on wildtype and clone-specific genomic DNA.

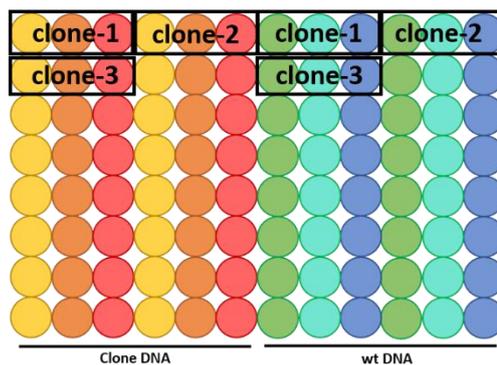


PRIMER SEQUENCES

cloneID-uni:	clone specific; see QC sheet
cloneID-opposite:	clone specific; see QC sheet
GT-universal:	GAGCCAGAACCAGAAGGAACTTGAC
LV-alternative:	AGAGCTCCTCTGGTTTCCCTTTC
RV-alternative:	TACAGACGCAGGCGCATAACATC
T2GT-alternative:	AAGGTCAGTGCAGGATCTGCTGAA
T2pA-alternative:	CCGGGCAATGGATTGATATTGCG

PCR REACTION

- Prepare the left half of a 96well PCR plate and provide 1µl of each primer (20µM) following the scheme below (total volume/well 2µl, only yellow, orange and red wells). The reactions for one clone should be in the same row (clone 1). For additional clones, you should either use adjacent columns (clone 2) or the row below (clone 3).



cloneID-uni + GT-universal	yellow + green
cloneID-uni + cloneID-opposite	orange + cyan
cloneID-opposite + GT-alternative	red + blue

- Dilute the required amount of 2x GoTaq Green Mastermix (Promega M712) with dH₂O and keep it on ice. For every clone you have 6 reactions. e.g. for 2 clones = 12 samples → prepare 13x mastermix.

	1x	13x
2 × GoTaq Green Mastermix	12,5µl	162,5µl
dH ₂ O	9µl	117µl
	21,5µl	279,5µl

- Add 43µL of the mastermix to each well on the left half (yellow, orange and red wells) containing primers. Total volume/well: 45µl.
- Use a multichannel pipet to transfer 22,5µl of each well (primers + mastermix) to the corresponding wells on the right half of the plate (yellow → green; orange → cyan; red → blue) to create duplicates.
- Add 2,5µL of the corresponding genomic DNA to the wells:
 yellow, orange, red → clone genomic DNA
 green, cyan, blue → wildtype genomic DNA
 Total volume of each PCR sample/well: 25µl

Biorad C1000 cycle parameters:

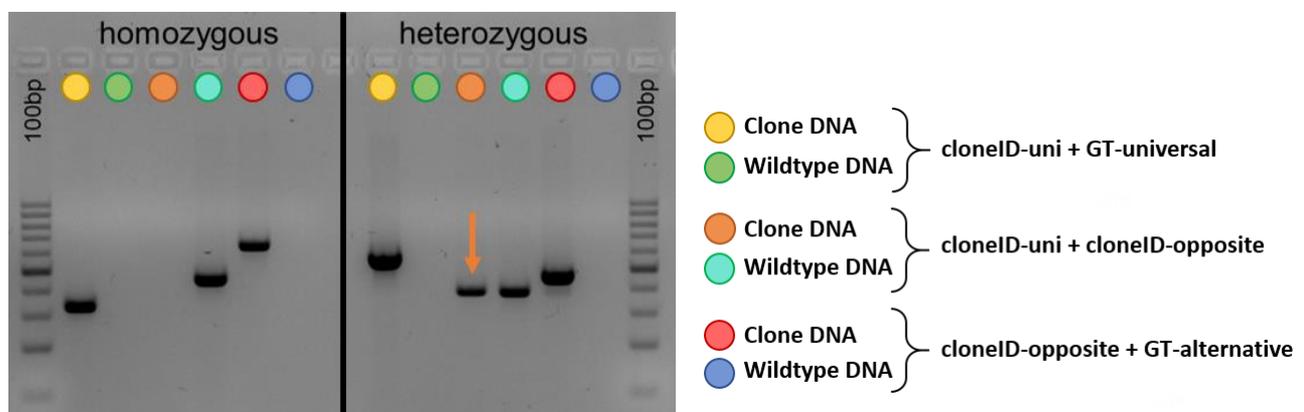
	2 min	} × 35
95°C	30 sec	
60°C	60 sec	
72°C	60 sec	
72°C	5 min	
4°C	∞	

AGAROSE GEL ELECTROPHORESIS

- Load the PCR reactions on an agarose gel, load 100bp DNA Ladder on the outermost lanes.
- Always load the 6 PCR reactions of one clone together, so that the same primer pairs are next to each other i.e. **Yellow, Green, Orange, Cyan, Red, Blue** for each clone. You can use a multichannel pipet (for 28-lane combs) and load yellow/orange/red and green/cyan/blue in an intercalating manner.

ANALYSIS OF PCR RESULTS

On basis of the products of the different PCR reactions you can confirm the integration of the gene trap in correct location as well as assess the status of homo- or heterozygosity of this integration. If the PCR worked correctly and everything is fine with the clone you should get a result of the gel electrophoresis as shown in the example below.



Yellow and Red line: Using one primer flanking the locus of integration in combination with a genetrapp-internal primer (cloneID-uni and GT-universal or cloneID-opposite and GT-alternative) on the clone-DNA you should obtain a single PCR band per lane, confirming that the genomic location of the genetrapp was mapped correctly.

Green and Blue line: Using one primer flanking the locus of integration in combination with a genetrapp-internal primer (cloneID-uni and GT-universal or cloneID-opposite and GT-alternative) on the wildtype-DNA you should not obtain any band as there should be no genetrapp in the wildtype cells.

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Orange line: Using the primers flanking the potential genomic integration site on gDNA from the respective clone (cloneID-uni + cloneID-opposite) you will not obtain a PCR band as the mutagen is too large for PCR amplification. If you get a band here, the clone may be heterozygously targeted.

Cyan line: Using the same primer pair on wildtype genomic DNA (cloneID-uni + cloneID-opposite) you should obtain a single PCR band, confirming that that gene specific primers work.