



## Experimentation with Animals: A Key Aspect of the 3Rs: The Genetic Quality

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### Abstract

The genetic quality of laboratory animals is essential for reproducibility of scientific research. Working with animals of certified genetic quality is still a pending issue in Argentina due to the lack of routine genetic controls, of information on the genetic background of animals and of proper training. Apart from being concerned with having their results published and getting funding for research, scientists should know the genetic origin of laboratory animals. Consequently, they should perform genetic controls to verify whether animal integrity has been compromised by accidental genetic contamination or genetic drift. The aim of this work was to evaluate the genetic purity of the inbred C57BL/6J mouse strain from three animal facilities belonging to the Buenos Aires University School of Medicine network by analyzing a panel of microsatellite markers. Female mice tail samples (3-5 mm) were taken and genomic DNA was obtained by organic extraction. The genetic profile of each animal was determined by PCR-fragment analysis, using microsatellites D1Mit155, D2Mit493, D3Mit49, D13Mit13, D6Mit8 and D12Mit12, located on six different autosomal chromosomes and selected from the Mouse Genome Informatics database ([www.informatics.jax.org/searches](http://www.informatics.jax.org/searches)). The results obtained provided key data on the genetic quality of the three inbred animal colonies studied. They also served as an example for other laboratory animal facilities in Argentina and as a starting point to modify the conditions and management of laboratory animal colonies. We determined the genetic purity of the inbred C57BL/6J mouse strain in all animal facilities evaluated. All six loci analyzed were homozygous, certifying their isogenicity and phenotypic uniformity. These results are promising for animal facilities mainly performing biomedical research. They also show a positive evolution in handling animal colonies and use of the 3Rs, and researcher commitment with animal science, since they promote the supply of genetically quality-controlled animals. The positive impact of these results should encourage other researchers using this inbred strain to perform periodic genetic monitoring, thereby consolidating the supply of quality-controlled mice. This pioneering study carried out in IGEVEVET (CONICET- UNLP) should consolidate the genetic monitoring of inbred strains throughout the country.

**Keywords:** 3Rs; C57BL/6J; Genetic Monitoring; Conventional Animal Care Facility

### Abbreviations

AACyTAL: Argentine Association of Laboratory Animal Science and Technology; CICUAL: Committee for the Care and Use of Laboratory Animals; MINCyT: Ministry of Science, Technology and Productive Innovation; SNCF: National System of Animal Care Facilities; SSLPs: Simple Sequence Length Polymorphisms; PCR: Polymerase Chain Reaction; DNA: Deoxyribonucleic Acid; UBA: University of Buenos Aires; INTA: National Institute of Agricultural Technology; SPF: Specific Pathogen-Free; MGSC: Mouse Genome Sequencing Consortium; IGEVEVET: Institute of Veterinary Genetics; CONICET: National Scientific and Technical Research Council; UNLP: National University of the Plata; C57BL/6J: Inbred Sub Strain; B6: Abbreviation of C57BL/6 Inbred Strain; 3Rs: Replacement, Reduction and Refinement.

### Introduction

From the beginning of the last century to the present day, the laboratory animal is a fundamental part of biomedical sciences. It is used as a model to investigate and understand the diagnostic causes and treatments of diseases that affect human beings. Laboratory mice represent approximately 80% of the demands of animal models worldwide [10]. Throughout history, great discoveries were only possible through the use of laboratory animals. Albert Sabin, who developed the polio vaccine, said, "Without animal research, polio would still be claiming thousands of lives each [11]. However, it is necessary to educate about the use and management of laboratory animals. In 1959, the principle of the 3Rs was formulated, in response to the growing experimentation with animals and in many cases their misuse and care. The 3Rs stand

for Replacement, Reduction and Refinement. In biomedical research, laboratory animals (genetically standardized mice) cannot be dispensed with. Since the creation of the first consanguineous strain (C57BL/6) in 1921 and with the creation of the first laboratory animal production centers in 1947 and 1963, the demand for laboratory animals has grown exponentially. Currently, there is a large number of genetically defined lines, such as consanguineous (inbred strains) and congenital, which include thousands of mutations and chromosomal rearrangements [6]. In this sense, one of the 3Rs principles [12] is pointed out: the reduction in the number of animals without losing precision. This is achieved with genetically homogeneous animals.

The advantage of working with consanguineous lines of mice is concerned with important characteristics such as genetic equality and phenotypic uniformity, which are the axis of the reproducibility of experiments. On the other hand, homozygosis allows comparing a single variable between a control and an experimental group, and attributing any difference in a parameter to that variable.

Lines with more than 200 generations of inbreeding, as is the case of the classical laboratory lines C57BL/6, C57BL/10, C3H, CBA and BALB/c, have 100% consanguinity coefficient (all loci are expected to be homozygous). Among the inbred mouse strains used in biomedical research, C57BL/6 (B6) is the most cited and best characterized. For that reason, in 1999 this strain was selected by the Mouse Genome Sequencing Consortium (MGSC) to represent the reference genome of the "laboratory mouse" [5], and is also the background strain of Knockout mice.

The main animal producers globally are The Jackson Laboratory and Charles River, founded in 1947 and 1963, respectively. Both lead the market for laboratory animals worldwide, promoting different educational programs on the good use and handling of laboratory animals. These centers are located in the USA and have branches in most European countries, but not in Latin America.

In Latin America, laboratory animals are provided by the two mentioned centers, which represents difficulties for their regular import and high import cost. In Argentina, different centers produce laboratory animals. They are classified according to the presence or absence of pathogenic organisms into specific pathogen-free (SPF) facilities and conventional (free of infectious organisms to man) ones. These production centers provide animals for small breeding and experimentation animal facilities belonging to different institutions in the country (universities, institutes of CONICET, INTA). All of these animal facilities share a common reality, which is the lack of genetic monitoring and the absence of animals with certified genetic quality from local laboratory animal suppliers.

In an endocrine system, opposing forces act: genetic drift, as a consequence of the spontaneous mutations that occur in consanguineous strains. It was estimated that a phenotypic mutation occurs every 1.8 generations of breeding [2]. A strain can carry undetected mutations for years, unless they have phenotypic manifestations. The risk of spreading a mutation is greater in a small colony than in a large one, since the probability of using a mouse carrying the mutation as a player is greater in a small colony. Based on the situation described above, the objective of this study was to survey a colony of inbred C57BL/6J mice from three conventional animal facilities belonging to the School of Medicine of the University of Buenos Aires (UBA) and evaluate the consanguinity system, namely, phenotypic uniformity, homozygosis and characteristics of the consanguineous lines, by analyzing a panel of microsatellite markers. The microsatellite analysis based on the PCR technique has considerable advantages over biochemical and immunological methods as a tool for the genetic monitoring of pure strains, especially in developing countries. It is an economic technique and, in addition, the selected microsatellites are polymorphic among laboratory strains.

This study was complemented by a survey that collected data on the management of animal colonies grown and maintained at the UBA School of Medicine, where genetic monitoring has been absent for many years. Having experimental animals with controlled genetic quality is a current problem in the context of animal facilities in Argentina. Thus, this study was conceived to define the genetic quality of laboratory animals and provide data on the genetic origin of the strains with which they have been working for years. In this way, it seeks to promote genetic control within the scope of the School of Medicine of the UBA, with the challenge of making it extensive nationwide.

## Materials and Methods

DNA was extracted from the tail tips (3-5 mm) of three adult female C57BL/6J mice (5 months old) selected from three of the breeding pairs used to establish the conventional colony in each animal facility. The animals were raised under controlled environmental conditions of temperature (21-23 °C), humidity (70%) and light (12:12 light: dark). Commercial granulated food (Cooperation, Buenos Aires, Argentina) and filtered water were provided.

Genomic DNA was obtained by organic extraction, by digestion with proteinase K (10mg/ml) and DTT (0.5M) in S digestion buffer, and incubation at 55 °C overnight. Chloroform was then added and samples were centrifuged at 14,000 g for 5 min twice. The upper aqueous phase was preserved, separated and ammonium acetate was added and centrifuged at 14,000 g for 5 min. The supernatant was transferred into a new tube and 100% precooled ethyl alcohol

was added until the tube was complete. The tube was left at -20 °C for 1 h; then it was centrifuged at 14,000 g for 15 min, and the contents were gently turned over. The pellet was washed with 1ml of 70% cold alcohol, and allowed to dry at room temperature. Finally, the resulting pellet was resuspended in 50-100 µl of sterile water overnight at 37 °C. The DNA concentration obtained was measured by spectrophotometry.

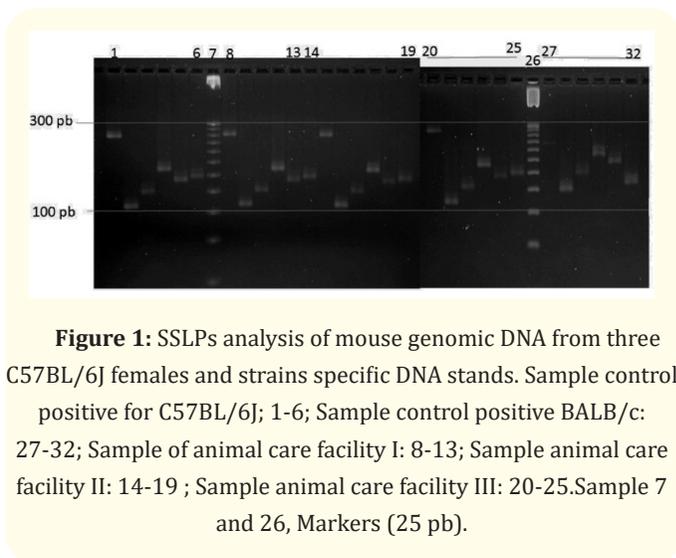
To discriminate between the genetic material associated with C57BL/6J and BALB/c, six microsatellite loci were analyzed: D1Mit155 (chromosome1), D2Mit493 (chromosome2), D3Mit49 (chromosome3), D13Mit13 (chromosome 13), D6Mit8 (chromosome 6) and D12Mit12 (chromosome 12). Primers were selected from the mouse genome information database (MGI).

The PCR reaction was performed in a final volume of 25 µl, which contained 1.5 mM MgCl<sub>2</sub>, using thermal cycler Axygen (USA). The amplification program included an initial denaturation of 94°C for 2 min, followed by 40 cycles of 35 sec at 94°C, 45 sec at 55 - 61°C and 45 sec at 72°C, and a final incubation at 72°C for 5 min. The amplification products were analyzed by electrophoresis in 4% agarose gels in 0.5X Tris-borate-EDTA buffer, ran at 120 volts for 2 h. The bands were detected by staining with ethidium bromide in UV light. A molecular basis marker of 25 base pairs (Promega, WI, USA) was used. In addition, the study included DNA controls belonging to strains C57BL/6J and BALB/c, from the Laboratory of Experimental Animals of the School of Veterinary Sciences of the National University of La Plata, descendant of mice purchased at The Jackson Laboratory.

The surveys carried out in each animal facility included questions such as quantity and type of strains kept, number of couples bought to refresh the colony, number of animal suppliers, number of people working and whether they have received any training in the use and care of laboratory animals, and if they were enrolled in the National System of Animal Care Facilities. Based on this analysis, the results obtained were compared.

### Results

The results obtained of the six loci analyzed are shown in Figure 1. Markers of the samples from the three animal facilities studied coincided with the genetic profile of the DNA control corresponding to strain C57BL/6J. The six microsatellites(SSLP), were homozygous for the six markers present in the following genotypes: D1Mit155 (252 bp), D2Mit493 (109pb), D3Mit49 (128 bp), D13Mit13 (148 bp), D6Mit8 (164 bp) and D12Mit12 (146 bp) (Table 1). These results confirmed that there was no genetic contamination with another strain.



**Figure 1:** SSLPs analysis of mouse genomic DNA from three C57BL/6J females and strains specific DNA stands. Sample control positive for C57BL/6J; 1-6; Sample control positive BALB/c: 27-32; Sample of animal care facility I: 8-13; Sample animal care facility II: 14-19 ; Sample animal care facility III: 20-25. Sample 7 and 26, Markers (25 pb).

Chromosome	Position (Mb)	Locus	Size (bp)		
			BALB/c	C57BL/6	
1	194	D1Mit155	216	252	forward: ATGCATGCATGCACCGT reverse: ACCGTGAAATGTTACCCAT
2	153	D2Mit493	127	109	forward: GTCTCTACCTGAGTTCCATCACA reverse: TCCCGAGTTGTCCTCTATG
3	89	D3Mit49	148	128	forward: CTTTTTCGCGCCACTTTC reverse: TCCTTTTAGTTTTTGATCCTCTGG
6	83	D6Mit8	190	164	forward: TGCACAGCAGCTCAATCTCT reverse: GGAAGGAGGAGTGGGGTAG
12	24	D12Mit12	170	146	forward: TTCAATGCCTCTGGCTTCT reverse: GATTACCGGTTGTGACCT
13	56	D13Mit13	138	148	forward: CTGTGGTAAGTCCAGATTTC reverse: GGAAAGAGTAGGAAGATGCC

**Table 1:** Primers selected from the mouse genome information database Mouse Genome Information database.

The surveys on the management of the colonies analyzed, we did not find significant differences in the answers of the three animal facilities surveyed. Similar results were obtained concerning personnel who had received regular animal laboratory training. Despite only two animal facilities had joined the National System of Animal Care Facilities, all three were committed to and supported the training of their staff, even without being part of the National System.

### Discussion and Conclusion

Genetic monitoring is an essential tool for the management of mouse colonies. The lack of authenticity of strains has been a long standing problem in animal facilities. Since the creation of the National System of Animal Care Facilities in 2013, the provision of animals with certified genetic quality continues to be a pending issue [1], mainly due to the lack of legislation on laboratory animals lending support to the control of such genetic quality. Genetic controls are under the decision of each animal facility. However, there is a strong demand on the part of scientific journals about the publication of works that include tests with laboratory animals. In this sense, authors are required to know the genetic origin and the

origin of the animals used in their research, to compete and live up to the different international research centers. From this point of view, researchers who aspire to publish in renowned scientific journals have decided to use animals that perform routine genetic controls, and therefore have certification of their genetic quality. These analyses had to be carried out outside the country because there were no such services in Argentina, and their cost could not be faced by all institutions.

Based on this situation, it was proposed to perform the genetic control and monitoring of inbred strains and to promote national projects to give an answer to the problem of lack of genetic control in consanguineous strains. In order to fine-tune the technique and launch this project, a study was carried out in 1999 in a strategic place of biomedical research, the UBA School of Medicine, at the animal care facility of the National Academy of Medicine, where contamination of C57BL/6J strains with BALB/c was detected [7]. In the same year, another study was carried out on building conditions and the training of personnel among other issues [8]. It was concluded that 28 of the 31 animal facilities analyzed were inadequate, 2 were good and 1 had optimal conditions. In 1999, the network of Committees for the Care and Use of Laboratory Animals (CICUAL, for its Spanish acronym) [3], the Ministry of Science and Technology and Productive Innovation (MINCyT) and the National System of Animal Care Facilities had not been created [4].

In 2019, we carried out a new study in the scope of the UBA School of Medicine, finding different results from those obtained in 1999. In all the animals analyzed, the six loci were homozygous for the expected alleles, keeping the characteristics typical of the consanguineous line C57BL/6J: genetic equality, phenotypic uniformity and homozygosis. These characteristics are the basis for the reproducibility of experiments and provide scientific validity to the results obtained with animals that have undergone genetic monitoring. These results are encouraging for researchers and institutions and for the performance of regular genetic monitoring of strains.

On the other hand, in the context of consolidated organizations promoting education and dissemination of the 3Rs, such as the Argentine Association of Laboratory Animal Science and Technology (AACyTAL) and the CICUAL network, the Ministry of Science and Technology and the National System of Animal Care Facilities have managed to invest in training and infrastructure, adding to a positive evolution in animal facility daily work. Furthermore, it was possible to assess the positive impact of training and the commitment of researchers and officers, encouraging the promotion of genetic strain monitoring and investment from national organizations so that animal facilities throughout the country can have access to these important controls.

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## Conflict of Interest

The authors declare no conflict of interest.

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