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CHAPTER

31

Genetic Monitoring of Laboratory Mice and Rats

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OUTLINE

I. INTRODUCTION

In the early days of research involving mice and rats, the importance of both genetic and health quality and monitoring of that quality was not recognized. The health quality of animals was quickly reflected in sick animals that could not be used for research, so immediate strides were made to ensure animal health in the research environment. Recognition of and efforts to maintain genetic quality lagged behind as laboratory animal genetics was initially only important to geneticists. As detailed investigations into genetics and immunology

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began, the inbred mouse and more recently, the rat, arose as genetic models of disease (Malakoff, 2000) and with those came the challenge of maintaining genetic homogeneity. Human error and genetic drift are ever-present sources of unwanted genetic heterogeneity, which ultimately lead to experimental irreproducibility. Genetic monitoring and colony maintenance quality control are necessary measures to detect and remove unwanted genetic heterogeneity. This chapter will discuss the rationale for genetic homogeneity and monitoring, the history of genetic monitoring from its inception to today, and current and historical means by which this may be accomplished. Also addressed in the chapter are current monitoring paradigms, or genetic quality control (GQC), for mice and rats, including inbred, congenic, outbred, and genetically engineered strains, as well as the role of breeders, vivaria, and end users in GQC. Finally, the future of genetic monitoring and GQC will be discussed.

II. HISTORY OF GENETIC MONITORING

In 1909, Clarence Cook Little created the first inbred mouse strain, DBA, at the Bussey Institute at Harvard University while at the same time, Helen Dean King created the first inbred rat strain, PA, at The Wistar Institute (Jacob and Kwitek, 2002). Since then, the number of genetically modified animals used in research has risen exponentially and their use has spread far beyond genetics and oncology research into almost every aspect of science and medicine. Today, scientists routinely use inbred, outbred, congenic, conplastic, recombinant inbred (RI), spontaneous mutant, and genetically engineered animals, as well as various combinations of the different types (Tables 31.1 and 31.2).

Loss of genetic homogeneity is a fairly routine occurrence throughout the history of the inbred mouse and rat. Human mistakes and mismanagement, murine size and abilities, and the genetics of coat colors, have all led to the inadvertent mixing of murine genetic material that researchers were attempting to separate and define. In 1974, the importance of genetic monitoring in commercial strains was demonstrated by Festing using mandibular measurements to determine genetic background (Festing, 1974). Roderick *et al*., in 1971 proposed using isoenzyme alleles to genetically monitor animals. Although contaminations certainly occurred before then (see the history of the C57BL/Ks [Naggert *et al*., 1995] and Groen's 1977 studies [Groen, 1977] for examples), the issue of genetic contamination of inbred mouse strains became a reality to many scientists in 1982 with a publication reporting contaminated BALB/c mice (Kahan *et al*., 1982). Festing reported several additional previously unpublished strain contaminations (Festing, 1982), including contaminated AKR mice, 'inbred' rats that were not histocompatible, and NZB mice contaminated within a research facility. More recently, Dahl salt-sensitive and Buffalo rats were found to be contaminated by other rat strains (Jones *et al*., 1994; Lewis *et al*., 1994; St Lezin *et al*., 1994) and the 129 family of mouse strains was discovered to be extensively genetically contaminated (Threadgill *et al*., 1997). Additional contaminations in mice have been reported in 101, SJL, and C57BL/6N (Benavides, 1999; Nitzki *et al*., 2007; West *et al*., 1985). Genetic contamination is not limited to inbred strains,

TABLE 31.1 Common Rodent Strains/Lines/Stocks

Strain	Description
Inbred	Strain established by a minimum of 20 consecutive generations of sister × brother matings (sib matings)
	Maintained by sib mating (http://research.jax.org/grs/type/inbred/index.html)
Outbred	Stock/line established and maintained by strict avoidance of sib mating
	Commonly, maintained using a defined breeding scheme and multiple breeding lines
F1 hybrid	Created by mating mice of two inbred strains in a defined direction – i.e., the B6D2F1 hybrid mouse line is produced by mating a C57Bl/6 female to a DBA/2 male at each generation (http://research.jax.org/grs/type/hybrid/index.html)
F2 hybrid	Created by mating two F1 hybrid mice – i.e., B6D2F2 is produced by mating 2 B6D2F1 animals
Recombinant inbred	Lines established by inbreeding of the progeny of individual F2 hybrid sibling pairs for at least 20 generations (http://www.jax.org/smsr/ristrain.html)
Congenic	Introduction of a mutation onto a defined inbred strain background by 5–10 or more generations of matings back to the inbred strain with selection for the mutation (backcrossing)
	Established congenic strains generally are maintained by purely sibling mating
Consomic	An inbred strain that carries one chromosome from another inbred strain
	Ten generations of backcrossing and selection for the donor chromosome is required for a fully consomic strain
	Mouse consomic panels generally consist of 21 strains, each carrying a different donor chromosome (1–19, plus X or Y) on a single host inbred strain background (http://research.jax.org/grs/type/consomic.html)
Conplastic	An inbred strain that carries the nuclear genome of one inbred strain and the cytoplasm and cytoplasmic (mitochondrial) genome of another
	Since the egg contributes the embryonic cytoplasm, 10 generations of backcrossing inbred females to an inbred male recipient is required for a fully conplastic strain (http://research.jax.org/grs/type/conplastic.html)

TABLE 31.2 Common Mutations Maintained in Rodent Colonies

Mutation type	Description
Spontaneous	Genomic alterations that arise generally from errors in the normal cellular processes, such as DNA replication, mitosis, or meiosis, chromosome structure changes, etc.
Induced	Genomic alterations that arise from intentional exposure to agents that can affect the normal cellular processes or genome structure, such as mutagenic chemicals or various forms of radiation
Genetically engineered – transgenic	Random insertion of DNA sequences into the rodent genome
	Transgenic rodents generally are created by microinjecting purified DNA into the nucleus of a fertilized egg; the introduced DNA contains all or part of a gene's coding sequence, usually with gene expression control elements
	Commonly generated to examine the expression of a foreign gene or overexpression an endogenous mouse or rat gene
Genetically engineered – gene targeted	Precise integration of DNA sequences into the rodent genome by homologous recombination
	Gene targeted rodents are commonly created by introducing DNA with targeted changes into embryonic stem (ES) cell genome in vitro, then microinjecting altered ES cells into host blastocysts to create a chimeric mouse
	Gene targeting can be used to ablate a gene ('knock-out'), to replace a gene ('knock-in'), or to regulate the temporal or spatial expression of a gene ('conditional mutation') by placing it under control of inducible elements
DNA sequence-specific genomic editing	Sequence targeted genomic alterations (point mutations, insertions, deletions, etc.) created directly in cells, embryonic stem cell or fertilized eggs by using zinc finger nucleases (ZFNs), sequence specific RNA-guided endonucleases (CRISPR-Cas9) or transcription activator-like effector nucleases (TALENs)
	These technologies decrease the requirement for extensive breeding to establish a mutant mouse strain on a consistent genetic background (http://jaxmice.jax.org/news/2014/CRISPR_Onestep.html)

however, as contaminations have been reported in MHC-congenic mice and RI mice as well (Marshall *et al*., 1992, Nandakumar and Holmdahl, 2005). These incidences of genetic contamination, and the many others that go unnoticed or unpublished, underscore the need for standardized genetic monitoring.

One of the oldest methods of genetic monitoring, and one in use to this day, is simply verification of normal characteristics for the strain in question. Examples of observable, expected phenotypes include coat color, body size and shape, reproductive performance, and behavior. To use coat color as an easily accessible example, it is easy to confirm that inbred albino animals, which are by definition homozygous for recessive *Tyr* alleles, have not been contaminated by a pigmented strain (e.g., this colony of BALB/c mice is comprised entirely of albino animals, therefore there has not been a contamination with a pigmented strain). Scientists and veterinarians should know the basic phenotypes of the strains with which they work, or know where to quickly obtain this information if abnormal phenotypes are seen and genetic contamination is suspected.

Beyond simple monitoring of observable, expected phenotypes, more sophisticated means of GQC were developed as the use of laboratory rodents expanded into various fields of study. Soon after the discovery of the major histocompatibility complex, tail skin grafting was developed as a way to ensure that animals were genetically identical (Whitmore *et al*., 1996; Bailey and Usama, 1960). Animals were anesthetized and skin grafts between animals of the same sex (or female to male animals; the reverse being ineffective due to the presence of antigens produced by genes on the Y chromosome) were placed on the ventral surface of the tail. If animals rejected the skin grafts, then there were major or minor histocompatibility differences and, therefore, they were not the same strain or substrain. For those who wanted to monitor genetic quality of their animals but did not want to perform surgery, morphometry, most specifically mandibular measurements taken after the animals were euthanized, was also used to ensure that animals were genetically identical (Lovell *et al*., 1984; Festing, 1972, 1993).

From 1960, when the inbred strains were known only by coat colors and MHC, to 1971, when the use of biochemical markers began to be widespread, inbred strain characteristics became more clearly defined. Biochemical loci rarely showed more than two or three allelic forms in inbred mice and by monitoring the forms of isoenzymes, a biochemical 'fingerprint' for each strain could be easily determined using blood, tissue extracts, and urine. Although more than 25 different standard biochemical markers were devised, The Jackson Laboratory found that a panel of approximately 10, combined with coat color, could distinguish one strain from the approximately 100 others maintained in their Foundation Stocks colony (see Fig. 31.1; Fox *et al*., 2007). The weakness of this approach is its lack of granularity; although the isoenzymes examined allowed for strain discrimination for these biochemical variants, more subtle differences or contaminations might not be detected. In addition, animals must be euthanized to examine biochemical markers and tissue. However, no reasonable option was available until direct examination of DNA became feasible on a large scale.

In some cases, especially for some immunologic congenic strains, where the only difference between animals

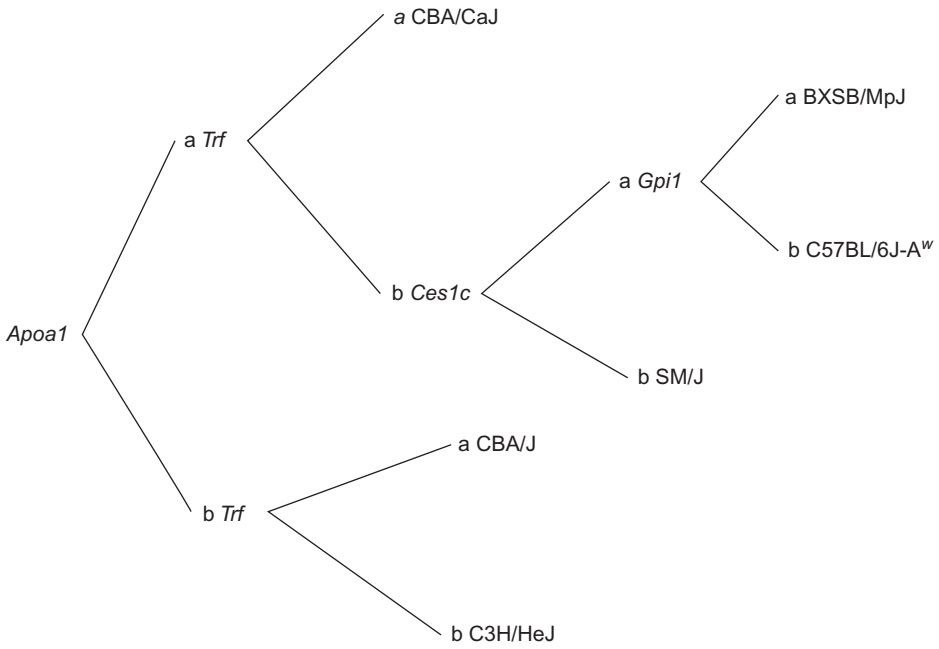

FIGURE 31.1 An illustration of how isoenzymes can be used to differentiate a group of mice with the same coat color. Isoenzyme genes and alleles are in italics; mouse strain names are in normal text. Based on the alleles present, animals can be differentiated–for example, a CBA/J will have *Apoa1*^{b} and *Trf*^{a}, while a C3H/HeJ will have *Apoa1*^{b} but *Trf*^{b}. This is a minimal amount of information or a critical subset of isoenzymes necessary to differentiate between the strains in the colony. Redrawn from Fox *et al.* (2007).

might be at the histocompatibility locus, immunologic markers are still in use. In immunologic tests, hemagglutination, in which the coagulation of red blood cells demonstrates the presence of incompatible alloantibodies, may be used to demonstrate that mice are expressing different red blood cell antigens than expected. A titer is determined by testing constant numbers of cells against serial dilutions of antisera and reporting the lowest dilution at which agglutination can be recognized. The primary means of demonstrating alloantibodies, however, is usually by examining white blood cells. If they are lysed or otherwise damaged by the presence of alloantibodies, they lose their ability to exclude a dye (Gorer and O'Gorman, 1956) or they refract glycerine differently (Shiroishi *et al.*, 1981). In some cases, there is only one assay that can distinguish one mouse from another. For example, only the hemolytic complement (Hc) assay can distinguish between the two congenic strains B10.D2-*Hc*^{1}*H2*^{d}*T18*^{c}/nSnJ and B10.D2-*Hc*^{0}*H2*^{d}*T18*^{c}/nSnJ, as the former expresses Hc while the latter does not.

Advances in the ability to manipulate DNA led to further sophistication in the way in which animals were monitored for genetic uniformity. The first method described was the use of DNA fingerprinting, or DNA profiling, to distinguish inbred strains (Russell *et al.*, 1993). DNA fingerprinting originally relied on the use of restriction fragment length polymorphism (RFLP), in which DNA was isolated, digested using bacterial restriction enzymes, and then Southern blotting was used to detect the RFLPs. This technique was effective, but cumbersome. With the discovery and widespread adoption of PCR as a tool for DNA investigation, RFLP analysis was quickly supplanted by PCR amplification of microsatellite repeats, short repeating sequences of 2–6 base pairs of DNA (also known as simple sequence length polymorphisms [SSLPs] or short tandem repeats [STRs]). Microsatellite panels were developed for mice and rats, and the use of SSLPs to analyze murine DNA for genetic contamination was widespread until recently and is still in use today (Montagutelli *et al.*, 1991; Hirayama *et al.*, 1994; Kloting *et al.*, 1995).

Current genetic monitoring using DNA relies on single nucleotide polymorphisms (SNPs) (Wiltshire *et al.*, 2003; Petkov *et al.*, 2004a,b; Fox *et al.*, 2007; Bothe *et al.*, 2011; Zurita *et al.*, 2011). This method of monitoring for genetic contamination was moved from theoretical to practical in 2004, with a publication by researchers at The Jackson Laboratory, aided by KBiosciences (Petkov *et al.*, 2004a). Petkov *et al.* developed a panel of 1638 SNPs, and then determined that a minimal panel of 29 SNPs was sufficient to monitor the genotypes of over 300 strains of mice maintained at The Jackson Laboratory (Petkov *et al.*, 2004b). The identification of SNPs has proceeded at a rapid pace since then, and commercial suppliers of SNP testing routinely offer panels of thousands of SNPs for genetic monitoring and linkage mapping. SNPs are also being routinely used to distinguish substrains as seen in Zurita *et al.* (Zurita *et al.*, 2011) and to monitor rats for genetic contamination (Nijman *et al.*, 2008; Saar *et al.*, 2008;

Bothe *et al*., 2011) SNP testing offers several advantages when compared to classical isoenzyme and tissue evaluations, including the sheer number of described variants, the ability to collect samples from living animals, and SNP analysis' ability to detect subtle genomic changes. In addition, SNPs can provide good genomic coverage and can be tailored quite closely to chromosomes or chromosomal portions of interest with high resolution.

While classical genetic monitoring paradigms are useful to detecting inadvertent, contaminating alterations in an animal's or strain's genome, an animal's or colony's genome can change over time for other reasons, such as the accumulation and fixation of spontaneous mutations and the resulting genetic drift (Casellas, 2011). Genetic monitoring may not be useful in these situations, unless the drifted or mutated region randomly coincides with a monitored locus or results in a detectable phenotype change. In the case of outbred animals, genetic monitoring is useful to ensure that the colony is maintaining heterozygosity over time. Genetic monitoring of outbred animals, however, would not necessarily detect every possible inbred strain contamination, since inbred strains and outbred stocks often originate from the same predecessors (Beck *et al*., 2000; Chia *et al*., 2005). Where genetic monitoring excels is in the detection of contamination of one inbred strain by another or an inbred strain by mutant strain.

For commercial breeders, the challenge is to ensure each colony conforms to genetic and phenotype expectations, while realistically being able to monitor only a subset of animals from a large production colony. Typically, breeders organize their colonies in a pyramidal fashion (Figure 31.2), for which only very small number of pedigreed founder animals can be sequentially expanded to generate breeding units that may not be pedigreed, to breeding animals selected arbitrarily from a large group. Genetic contamination at the bottom of this pyramid potentially will affect many more animals and, in turn, researchers than genetic contamination at the top of the pyramid. Therefore, all foundation stock breeders should be rigorously tested for their strain background and expected mutations, while smaller numbers of breeders and progeny animals are examined in the downstream colonies. Through the use of modern DNA testing methodology, as few as eight animals would detect a contamination present in 30% of a large breeding colony with a 95% confidence interval (ILAR, 1976; Dubin and Zeitz, 1991), if certain assumptions are made.

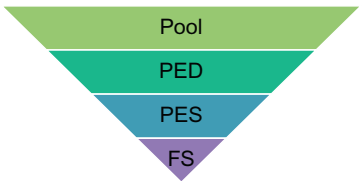

FIGURE 31.2 Organization of a large commercial production rodent colony. FS – foundation stocks: small number of pedigreed sibling pair matings, derived from cryopreserved stocks or progeny derived from a prior generation of FS matings; for individual strains, FS colonies are organized into multiple sibling-mated lineages; all FS breeders are genotyped for strain background and expected mutations, if applicable. PES – pedigree expansion stocks: expansion of pedigreed lineages by sibling pair matings of FS progeny, as well as progeny derived from a prior generation of PES matings. PED – production pedigree stocks: further expansion of pedigree lineages by two female × one male trio matings of FS, PED, and PED lineages. Pool or distribution colony: single generation of matings of progeny from FS, PES or PED colonies, all progeny placed into inventory. The illustrated colony plan can be adjusted to meet demands. Every strain/line/stock colony should have at least a pedigreed FS (or a supply of lineage-identified frozen animals) and a distribution colony. PES or PES/PED colonies are required only to generate the number of Pool colony breeders to ensure the desired supply of experimental or inventory animals.

III. RODENT STRAINS: GENETIC HOMOGENEITY OR GENETIC DIVERSITY

During cell division, spontaneous mutations naturally occur primarily during DNA synthesis due to incomplete replication-dependent repair. When spontaneous mutations occur in the germ line, the change is heritable and is the underlying cause of genetic drift. For the mouse, Drake *et al*. (1998) calculated a spontaneous mutation rate of 1.8×10^{-10} per base pair per replication, equivalent to 0.9 mutations per gamete per sexual generation. This natural genetic drift results in the acquisition of new alleles and phenotypes, previously not recognized in the strain.

To address the inevitability of spontaneous mutations and genetic drift, each commercial animal producer and rodent researcher should employ breeding schemes to ensure each strain, stock, or line remains consistent with genetic expectations. Does the colony's breeding plan conserve genetic homogeneity by limiting genetic drift or permit, even enhance, genetic heterogeneity by fostering genetic drift? In the simplest view, a spontaneous mutation creates a recessive, loss-of-function allele with no or little detectable phenotype expression when heterozygous. What is the impact of this heterozygosity on different mating schemes? Inbreeding decreases genetic diversity through the routine mating of siblings, expected to be among the most genetically homogenous individuals. However, sib matings also can enhance the probability of a heterozygous mutation becoming homozygous through the generations, eventually resulting in its detection as an overt or deleterious phenotype. A detectable phenotype permits the elimination of the new mutation from the colony by removing any affected and related individuals. By contrast, outbreeding or any approach that purposefully avoids the mating of related individuals will permit heterozygous spontaneous mutation to be carried in the

colony with little chance of phenotypic detection, promoting the genetic heterogeneity of an outbred population.

However, the greatest source of potential genetic divergence in any rodent colony is genetic contamination, incorrect matings or other undetected deviations. A GQC monitoring program should be designed to discover these natural and introduced errors before they are perpetuated by assessing not only the strain/stock/line genetic background, but also the presence and zygosity of specific alleles expected in specific colonies or individual mutant strains.

IV. SNP ANALYSIS: TESTING STRAIN BACKGROUND

For monitoring the strain background, the selection of SNP testing panels must take into account the extent of 'genetic insurance' that is acceptable and required. What depth and breadth of genomic coverage meets the reasonableness test by balancing the risk of genetic contamination with the colony's management procedures and practices, the effective deployment of resources, and the available analytical tools? In general, low-density SNP panels are most applicable for routine screening of a substantial number of large colonies. For example, all new breeders in The Jackson Laboratory's foundation colonies are screened with a panel of ~35 SNPs: these SNPs cover all the laboratory mouse autosomes and the X chromosome, with at least one diagnostic SNP per chromosome. This approach provides only a genetic snapshot of each colony, but a sufficient picture to differentiate all common inbred strains and most substrains. By contrast, high-density SNP panels are required for genome scanning, as well as for gene and quantitative trait locus (QTL) identification. The Mouse Universal Genotyping Array (MUGA, ~7851 SNPs) (Collaborative Cross Consortium, 2012), MegaMUGA (~80,000 SNPs; http://csbio.unc.edu/CCstatus/index.py?run=GeneseekMM), and the Mouse Diversity Array (~623,000 SNPs) (Yang *et al.*, 2009) are among the panels available for detailed genomic analysis. A variety of diagnostic SNP panels are available from commercial vendors or have been reported in the research literature. Further, community resources, such as the International Mouse Genome Informatics (MGI) website (http://www.informatics.jax.org/strains_SNPs.shtml) or the Rat Genome Database (http://rgd.mcw.edu/) catalog the SNP differences between inbred mouse and rat strains. Using these data, SNP panels can be developed to efficiently discriminate strains, lines or stocks or to conduct genome analyses.

When selecting a SNP panel, researchers must define their experimental goals, then balance the analytical costs against their desired outcome. The mapping of a mutation or phenotype to a specific gene or discrete chromosomal region likely will require high-density SNP panels or even genomic sequencing, whereas lower-density SNP panels generally will suffice to determine whether a strain is genetically contaminated.

When examining a strain, stock, or line's genetic background, it is critical to assess whether discrimination among inbred strain background is sufficient or whether the evaluation must be refined to differentiate among common inbred substrains. Substrains arise when animals from a common ancestor pair (i.e., an inbred strain) are independently sister × brother bred for at least 20 generations. For example, the development of the C57BL inbred strain began in 1921; by 1970, numerous C57BL substrains were recognized (Bailey, 1978). During the generations of independent breeding, a substrain accumulates a unique repertoire of spontaneous mutations and alleles, leading to substrain-distinct phenotypes. The widely used C57BL/6J and C57BL/6N substrains were separated in 1951 at generation F32, when The Jackson Laboratory transferred mice to the National Institutes of Health. The independent inbreeding of these mice at NIH resulted in the '*N*' substrain. In 1984, NIH cryopreserved this substrain at generation F124: the present C57BL/6NJ substrain is derived from the 1984 cryostock. By contrast, the present cryostock of C57BL/6J is at generation F226. Given that these two substrains have been independently maintained for ~300 generations, not all C57BL/6 mice are genetically or phenotypically equivalent and these differences can affect experimental results. Specifically, Simon *et al.* (2013) identified 36 coding sequence differences, as well as significant phenotypical differences, between the C57BL/6J and C57BL/6N substrains, two substrains separated for approximately 60 years.

V. ALLELE-SPECIFIC GENOTYPING: TESTING MUTANT STRAINS

For strains carrying known spontaneous, induced, or genetically engineered mutations, routine analysis of the presence and copy number of the mutant alleles is essential. Individual mutant strains, historically, were identified by an easily recognized phenotype, such as coat color. With the development of genetically engineered rodent strains, molecular confirmation of the presence and zygosity of engineered mutations became a critical GQC step, using a routine genotyping protocol is determined by the colony breeding requirements. Breeder genotype confirmation generally is sufficient for monitoring strains/lines bred homozygote × homozygote. By contrast, strains/stocks or lines with segregating mutations require genotyping of each progeny animal to identify the appropriate animals for subsequent breeding or experiments.

TABLE 31.3 Suggested Mating Schemes and Genetic Monitoring Strategies for Various Types of Animals Where Ensuring That Homozygosity and Genetic Uniformity Is Retained

Genetically homogenous				
		Options for genetic quality control testing		
Strain type	**Mating scheme**	**Which animals to test**	**Strain background assays**	**Allele-specific assays**
Inbred	Sister × Brother (sib mating)	Breeders	Homozygous for strain/substrain-specific SNPs	Can test for strain/substrain-specific alleles
Congenic or isogenic	Hom × Hom	Breeders	Homozygous for strain/substrain-specific SNPs	Homozygous for mutant allele
	Hom × Het (or reciprocal)	Progeny, including presumptive breeders		Homozygous or heterozygous for mutant allele
	Het × Het	Progeny, including presumptive breeders		Homozygous, heterozygous or wild-type for mutant allele
	Wild-type from colony (or inbred) × Heterozygous or hemizygous carrier (or reciprocal)	Progeny, including presumptive breeders		Heterozygous/hemizygous or wild-type for mutant allele
F1 hybrid	Inbred 1 × Inbred 2	Breeders	As for inbreds	As for inbreds
Established recombinant inbred (RI) lines	Sister × Brother (sib mating)	Breeders	Homozygous for line-specific SNPs	

VI. MAINTAINING STRAINS

Tables 31.3 and 31.4 provide options for maintaining common laboratory rodent strains/stocks/lines to ensure the desired genetic homogeneity or heterogeneity in the population, as well as for conducting GQC analysis to confirm colony status (see Berry and Linder (2007) for a comprehensive review).

A. Inbred Strains

Strains maintained by inbreeding provide the greatest assurance of genetic identity through sib mating at each generation. A strain is considered inbred after a minimum of 20 generations (F20) of sib matings; however, with continual inbreeding, the genetic homogeneity of the inbred strain background is further increased by 40 generations of inbreeding, a strain is mathematically 99.98% homozygous (Green, 1981; Silver, 1995). The common inbred laboratory mouse strains (C57BL/6, DBA/1, DBA/2, BALB/c, CBA, C3H) were developed early in the last century (1909–1921) and are presently at F200 or greater generations of inbreeding. The primary GQC consideration is genetic consistency of the inbred strain background. Inbred strain SNP analysis should exhibit homozygosity for all markers; however, strain/substrain specific alleles can be tested for a higher level of confidence and discrimination.

TABLE 31.4 Suggested Mating Schemes and Genetic Monitoring Strategies for Various Types of Animals Where Ensuring Heterozygosity Is Retained Is Paramount

Genetically heterogenous			
		Options for genetic quality control testing	
Strain type	**Mating scheme**	**Which animals to test**	**Assays to monitor background**
F2 Hybrid	F1 × F1	Progeny	Random distribution of grandparental inbred strain/substrain-specific SNPs
Established advanced intercross recombinant inbred (RI) lines	Sister × Brother	Breeders	Homozygous for line-specific SNPs
Outbred	Various (avoid sib matings)	Any	Random distribution of stock-specific SNPs
Random bred	Randomly generated among lines (avoid sib matings)	Any	Random distribution of population-specific SNPs

An additional approach to ensure the long-term genetic consistency of inbred strains is to combine sib mating with the cyclic reintroduction of cryopreserved animals and rebuilding of foundation colonies with pedigreed, cryopreserved embryos, thus ensuring downstream colonies and inventory mice are as genetically consistent as possible with the foundation stocks and substrains have not inadvertently arisen within the colony. The Jackson Laboratory's Genetic Stability Program (GSP, US patents 7,592,501 [2010] and 8,110,721 [2012]) provides a colony management approach for maintaining breeding colonies within a defined generation interval for many years or decades, thus limiting genetic drift and avoiding substrain divergence.

B. Isogenic Strains

Isogenic (coisogenic) strains differ from their parental inbred strain at a single locus, generally due to fixation of a spontaneous mutation initially recognized by an observable phenotype. When these mutant mice are inbred separately from their parental strain, the isogenic mice will retain their mutant phenotype, but will acquire background mutations not found in the parental strain. It is recommended that the mutant strain background is refreshed by backcrossing to the parental inbred strain approximately every 10 generations to ensure the mutant strain remains truly isogenic with the original parent strain. An alternative approach is to develop a stock of cryopreserved embryos derived from backcrossed animals and periodically refresh colonies with cryorecovered embryos (Taft *et al.*, 2006). When properly maintained, an isogenic colony will exhibit an identical strain background to its parental inbred strain, deviating only at the specific mutant allele. The mutant allele can be confirmed by the distinctive phenotype or by directly testing for the specific genomic change that created the mutant allele.

C. Congenic Strains

Congenic (and consomic) strains contain a discrete DNA region derived from another strain (for consomic strains, this region is an entire chromosome; Table 31.1). Historically, congenic mice were developed by the repeated backcrossing of strain-specific alleles onto another strain background with the selection of the desired allele/region at each backcross generation. With genetically engineered mutations, initially generated either by embryonic stem cell-mediated gene targeting or by the introduction of transgenic DNA constructs, the generation of congenic mice has accelerated, facilitating the characterization of individual mutations on a well-defined strain background and eliminating potential ambiguities due to variable strain background effects.

Generally, 10 generations of backcrossing is considered the standard for congenic strain development. Theoretically, with each backcross, the mutant stock acquires 50% of the inbred backcross partner's DNA sequences. By the 10th backcross generation (N10), 99.99% of the congenic strain background will be from the recipient inbred; only the congenic interval, the genomic region flanking the introduced allele, is essentially unchanged from the donor strain (Berry and Linder, 2007). However, theory and practice may not be in accordance and a genome scan is recommended to quantify the congenic strain background – the contributions of the donor and recipient strains – after repeated backcrosses. Marker-assisted backcrossing can accelerate the development of fully congenic strains by SNP analysis of progeny at each backcross generation and the selection of next-generation breeders for their content of recipient strain sequences (Berry and Linder, 2007; Wakeland *et al.*, 1997).

The optimal mating scheme for individual congenic lines will vary, generally determined by the phenotype of the introduced allele. The congenic strain background will remain isogenic with the recipient inbred strain, if the congenic colony is refreshed by periodic backcrossing or by the introduction of cryopreserved, backcrossed stocks. To ensure congenic strain identity, however, the presence and zygosity of the mutant allele should be routinely confirmed.

D. Genetically Modified Strains

Genetically modified strains (Table 31.2) are created through microinjection of DNA constructs (transgenic strains), targeted embryonic stem (ES) cells (gene targeted strains) or genomic editing through the injection of programmable endonucleases (e.g., zinc finger nucleases [ZFN], TALEN, CRISPR/Cas9) into embryos (Hsu *et al.*, 2014; Joung and Sander, 2013). Recent advances in embryonic stem cell derivation technology and site-specific nuclease technologies now make it possible to genetically engineer any murine strain. Genetic monitoring of the engineered alleles by PCR is essential to successfully maintaining these strains. However, additional GQC is recommended depending on the type of engineered strain.

Transgenic alleles generated by microinjection of DNA into embryos can be unstable because the injected DNA randomly integrates into the genome, frequently in multiple copies organized into an array. The stability of these arrays varies across generations; therefore, it is important to monitor transgene copy number and expression periodically. It is also important to perform GQC on engineered strains when they are first developed or acquired. This quality control depends on the nature of the engineered allele. A common quality control

issue faced by mouse repositories involves conditional alleles. Conditional alleles containing loxP sites require cre-mediated recombination, which is usually provided through a cross of the strain carrying the conditional allele with a strain carrying a tissue specific cre (usually a transgenic strain). Subsequent breeding, assisted by a cre-specific PCR assay, is required to remove the cre, but this step is often neglected and unwanted cre alleles remain segregating in the colony.

The development of mutant mice has been revolutionized by the use of programmable endonucleases to generate sequence-specific mutations *in vivo*. By microinjecting targeting vectors/nucleases directly into inbred strain zygotes (fertilized eggs or single cell embryos), specific mutations can be created on a defined strain background, obviating the requirement for repeated backcrossing to introduce the mutant allele onto a defined genetic background. However, researchers should be cautious in evaluating these progeny: initially, they may be mosaic for the introduced mutation or carry unwanted second-site mutations due to target sequence similarities. Therefore, sib mating, coupled with genotyping for the precise targeted mutation and survey of the strain background, are recommended to ensure these mice conform to desired expectations.

E. F1 Hybrids

F1 hybrid animals are generated by the mating of two different inbred strains at each generation, using the same the maternal and paternal inbred parents. The F1 animals are genetically homogeneous and heterozygous at every locus, with 50% of their genomes derived from each parent. While these animals cannot be maintained as continuously breeding strains, F1 hybrid progeny provide the advantage of heterosis or hybrid vigor with larger litters and larger pups with generally more robust constitution than either inbred parent. F1 hybrids, therefore, can provide a consistent genetic background for studying potentially deleterious mutations or detrimental agents. Since F1 hybrid progeny require the repeated mating of two inbred strains, the genetic identity of parental inbred strains must be unequivocal.

F. F2 Hybrids

F2 hybrid progeny arise from the mating of two F1 hybrid animals. As a result of meiotic recombination, F2 hybrid progeny are not genetically homogeneous; rather each F2 animal is genetically heterogeneous with an assortment of alleles, SNPs or other molecular markers derived from their inbred grandparents. F2 hybrid mice are often used as approximate controls for mutant stocks that are not fully congenic.

G. Recombinant Inbreds

RI lines are developed by the mating of randomly selected F2 hybrid progeny followed by sib mating to establish individual inbred lines. After 20 generations of inbreeding, individual RI lines will be genetically homogenous, but a family of related RI lines will exhibit considerable genetic diversity, reflecting the genetic backgrounds of F2 hybrid line founders (Crow, 2007). A family of related RI lines can provide a unique resource for mapping genes and QTLs (Pollard, 2012). The analytical power of related RI lines lies in their diversity, but care must be taken in ensuring individual RI lines remain genetically homogeneous by careful colony maintenance and GQC/SNP testing to ensure each line retains its distinctive strain genetic background. Periodic analysis for the expected strain parameters of individual RI lines should be conducted to elucidate whether each line maintains its anticipated characteristics or has acquired new phenotypes or acquired detectable genotype changes.

H. Advanced Intercross Lines

Historically, RI lines were developed by inbreeding F2 progeny derived from grandparent of two distinct inbred strains. More recently, the RI strategy has been augmented by the use of breeding schemes to maximize meiotic recombination in the RI population (Rockman and Kruglyak, 2008) or by using multiple inbred strains to create the hybrid founder animals (Aylor *et al.*, 2011; Philip *et al.*, 2011).

In 2002, the International Collaborative Traits Consortium began the development of RI lines that combined the genomes of eight inbred laboratory mice strains by using a funnel-breeding scheme (The Complex Traits Consortium, 2004; Collaborative Cross Consortium, 2012). Following three generations of funneled, 'hybrid' matings, individual RI lines were established by classic inbreeding for 20 generations, creating the Collaborative Cross (CC) RI lines. When fully inbred, each CC RI line is genetically homogenous, but individual lines contain a unique genome, incorporating contributions from the eight original inbred founders.

The chromosomes of each advanced intercross line contain a combination of alleles or chromosomal regions randomly derived from the founder lines through meiotic recombination. The use of high-density SNP panels and advanced analytical tools generally are required to develop a genomic map for each chromosome of individual inbred advanced intercross line, identifying which chromosomal regions and alleles are derived from each original founder lines. Periodic reconfirmation by high-density SNP analysis of continuously bred lines can determine whether the initial genomic map remains

essentially unchanged. Since any continuously bred line/stock will acquire spontaneous genomic changes, cryopreservation and recovery of the initially characterized lines can ensure genetic stability of individual lines. By comparisons among individual lines, specific traits can be mapped to specific chromosomal regions and, ultimately, correlated with founder line-specific alleles, strikingly illustrating the power of advanced intercross genomes for mapping genes, complex traits, and QTLs.

I. Non-Inbred Stocks (Outbred and Random-Bred Rodent Populations)

Rodents from outbred or non-inbred stocks are widely used for a variety of research applications that require animals with the greatest possible genetic variation (Aldinger *et al.*, 2009; Chia *et al.*, 2005; White and Lee, 1998; Hartl, 2001). When maintaining an outbred colony, the greatest challenge is ensuring the population, which may be split into several locations, truly retains its heterozygosity. The tendency of a closed colony built from a finite number of founder animals is to lose heterozygosity by the fixation of alleles over generations of continual mating: with increasing allele homozygosity, an outbred stock will become inbred. Therefore, outbred colonies must be maintained by a systematic breeding program optimized for breeding unrelated individuals (Nomura and Yonezawa, 1996; White, 1999) and by using methodology such as genomic scans employing high-density SNP panels to evaluate the extent of genetic heterozygosity. In general, outbreeding strategies require a large number of independent breeding lines with multiple breeding units for each line, unbiased breeder selection, and the routine infusion into the closed colony of animals from an independently maintained colony or cryopreserved stocks.

Another way in which heterozygosity and genetic diversity can be retained in colonies of outbred mice separated by space is by a scheme in which animals migrate to and from a founder colony (Charles River, 2011). In this means of preserving diversity, any drift or new mutations are retained and distributed to other colonies by means of the foundation colony. Animals from outlying colonies are rederived into the foundation colony at a rate of 5% of the foundation colony each year, and incorporated into the existing breeding scheme. The foundation colony is maintained through rotational mating. Each outlying colony also periodically receives 25% of its male breeders from the foundation colony as an efficient way of rotating alleles through a large population.

Random breeding provides another option for maintaining a population's genetic diversity (Aldinger *et al.*, 2009; Churchill *et al.*, 2012; Rockman and Kruglyak, 2008). The mating pairs at each generation are selected by use of a random number program, coupled with the exclusion of any randomly generated sib matings. The J:DO (Jax Diversity Outbred) mouse stock (Churchill *et al.*, 2012; Svenson *et al.*, 2012) is maintained by such a random mating scheme: the DO population was founded by the random mating of 144 partially inbred Collaborative Cross RI lines to establish 175 DO lines, which are randomly propagated at each generation. High-density SNP genome scans of DO progeny have confirmed the significant diversity of alleles among individual genomes and the utility of this stock for high resolution genetic mapping (Logan *et al.*, 2013; Svenson *et al.*, 2012).

VII. PHENOTYPE QUALITY CONTROL

GQC alone does not ensure a rodent model will meet experimental expectations and requirements. It is critical to know the expected phenotypes of any research strain and the impact of environmental or other factors on these phenotypes. Caging, diet, water, bedding, husbandry practices, and microbiome all may have a dramatic impact on phenotype. Both commercial providers and researchers should assess the physiological and biochemical parameters of the animals raised in their facilities and the potential impact of environmental changes on these variables. It is recommended that anyone receiving animals into a new location allow them to acclimate to their new environment prior to their use in experiments.

Commercial rodent provider public websites may provide basic phenotype information as well as other resources on the responses and experimental applications of individual research strains. The animal research community, moreover, has developed a wealth of databases that archive strain or mutation-specific genotypes and phenotypes. Among these are the Mouse Phenome database (http://phenome.jax.org/), the International Mouse Phenotyping Consortium (http://www.mousephenotype.org/), Mouse Genome Informatics database (http://www.informatics.jax.org/), the Rat Genome Database (http://rgd.mcw.edu/), and The National Bio Resource Project (http://www.anim.med.kyoto-u.ac.jp/nbr/phenome.aspx).

VIII. THE FUTURE OF GQC

The advent of high-throughput sequencing has revolutionized the field of genetics in innumerable ways. The sequencing and assembly of the mouse genome representing a single inbred strain, C57BL6, was completed

in 2002 (Mouse Genome Sequencing Consortium, 2002) after a three-year effort based on dideoxy or chain termination sequencing (a.k.a. Sanger sequencing). In 2011, The Sanger Mouse Genomes project published the whole genome sequence of 17 inbred strains using high-throughput/massively parallel sequencing demonstrating the power of this sequencing technology to generate DNA sequencing data on a scale that is orders of magnitude over earlier methods. Today, a single mouse genome can be re-sequenced to a depth of 30× coverage for less than $5000 using this technology (Yalcin *et al.*, 2012). Select regions of the genome can be subjected to high-throughput sequencing by applying any number of techniques that allow for enrichment of specific regions of interest. Selective sequencing is most commonly accomplished using a pool of DNA probes that share homology with the regions of interest. Enrichment for those regions is accomplished by probe hybridization to total genomic DNA in solution, followed by capture of the DNA hybrids and high-throughput sequencing. The most common application is selective sequencing of the coding portion of the genome or exome sequencing. Exome sequencing can be accomplished for as little as $900 and provides sufficient coverage to confidently genotype the coding portion of the mouse genome, which is ~50 Mb (Fairfield *et al.*, 2011). This method captures all of the variation within the annotated coding portion of the genome and can provide significant baseline data for any mouse strain. In fact, this approach is now used routinely as follow up GQC for spontaneous mutations that confer clinically interesting phenotypes in mouse colonies at The Jackson Laboratory (Fairfield *et al.*, 2011). Identification of spontaneous mutations also allows for the design of genotyping assays that can then be used to selectively remove these unwanted alleles from breeding colonies. While this approach is not yet cost effective for routine GQC, the race for the $1000 genome (Hayden, 2014) is driving costs down making high-throughput sequencing an eventuality that could have a significant impact on GQC options in the future.

IX. GQC: PRACTICAL ASPECTS

Genetic monitoring of rodent colonies focuses on molecular markers, biochemical or immunological assays to assess the allelic diversity within a colony, as well as individual genotypes. While these techniques provide qualitative and quantitative evidence of a colony's genetic constitution, the foundation of any GQC program lies with basic colony management and animal husbandry. These approaches are simple in concept and execution, but produce huge dividends in insurance of a colony's genetic status.

- Employ consistent, tested husbandry practices designed to avoid cross contamination between individual cages or strains.
- Segregate strains/lines/stocks by coat color or another observable phenotype.
- Use appropriate colony nomenclature and separate colonies with similar names. For example, separate C57BL/6J from C57BL/6N, BALB/c from BALB/cBy, etc.
- Consistently use distinct cage cards for each strain and separate strains with similar cage cards. Use the colony-specific cage cards for all cages, both breeders and weaned progeny. Employ colony management software coupled with bar-coded cage cards or other card or animal identification method. Among the available animal ID methods are standard ear notching or ear tags, bar-coded ear tags, microchip implants, or permanent tattoos.
- Consider maintaining pedigreed colonies: for inbred, isogenic, congenic or established RI lines, sibling breeder units are established at weaning, and each mating is recorded by family lineage in colony management software, on cage cards and in pedigree books or charts. While potentially far more complex, similar records should be kept for non-inbred colonies.
- Not only do pedigreed colonies reduce potential mating errors, but also lineage tracking provides an unambiguous record for tracking the ancestry of any phenotypic deviations, spontaneous mutations, or potential genetic contamination.
- Maintain physically separated colonies for each strain/stock/line. Commercial animal providers maintain their breeding colonies with several subcolonies (Figure 31.2): foundation stocks (FS) provide the breeders to expansion stocks that, in turn, send breeders to the distribution colonies. With independent colonies, spontaneous mutations or genetic contaminations can be limited to and eliminated from a single colony.
- Maintain cryopreserved stocks of each strain or population, using these stocks either to routinely replenish your foundation colonies, thus limiting genetic drift and substrain development, or to provide a source of new breeder stock in the event of genetic contamination or widespread fixation of new mutations (Wiles and Taft, 2010). Know the expected deviations of your strains, then detect, track and eliminate all phenotypic deviants whether expected or unexpected. Be on the alert for the recurrence of an unexpected phenotype within a strain or line.
- Animals exhibiting abnormal, unexpected phenotypes can result from the expression of a spontaneous mutation, a genetic contamination or

a developmental, often not heritable, mutation. The analysis of phenotypic deviants and their parents can elucidate the heritability and origin of the phenotype, facilitating the removal of undesirable contaminants before they become fixed in the colony.
- Supplement your colony management program with a consistent, statistically significant GQC program. For any colony, monitor the genetic background to ensure the desired composition is retained; for strains carrying mutations, monitor both the genetic background and the expected mutant allele(s).

When monitoring for spontaneous, induced, or engineered mutations,

- Employ allele-specific assays. While generic assays for drug resistance markers, Cre or green fluorescent protein provide a convenient option for routine genotype analysis, they do not provide the highest level of discrimination when a colony contains numerous similar strains or single strains carrying multiple alleles.
- Ensure allele-specific assays can unambiguously differentiate homozygotes from heteterozygotes from wild-type.
- For transgenic strains, for which the extent of a phenotype may be a function of the expression of multiple transgene copies, detection of the transgene alone may not be sufficient to ensure the strain's conformity to phenotype expectations. The use of assays to assess the transgene copy number may be essential to ensuring the stability and reproducibility of a transgenic model.
- Only accept unequivocal assay results, ascribable to individual animals. If any result is questionable, resample and repeat the allele-specific assay or remove the animal from your colony.
- Assess your process: where are the greatest vulnerabilities for breeding or using an incorrect animal? The greatest chance for confusion of individual animals or strains lies when animals move or are shipped from one location to another. Therefore, it is recommended that the identity of animals is confirmed when they arrive at their final destination or employ unambiguous animal or strain identification methods prior to movement.

X. CONCLUSIONS

Careful genetic monitoring of laboratory mouse and rat strains maintains genetic homogeneity, which is critical (with the exception of strains that are intentionally heterogeneous) for reproducibility in animal studies. Therefore, it is essential that the vivarium design and implement rigorous GQC protocols. GQC methods vary in their sensitivity and specificity as well as their cost; choice and implementation of GQC methods should be guided by the nature of the strains to be monitored. Finally, the responsibility of proper GQC is not only the responsibility of the vivarium, it should be carefully considered during strain development and by the end user to ensure responsible laboratory animal stewardship and high-quality scientific results.

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