WINTER 1992

Rodent Genetics and Genetic Quality Control for Inbred and F_1 Hybrid Strains, Part II

Genetically defined rodent strains with stable, identifiable phenotypes have played a central role in the advances made in biomedical research. Such strains have been developed by selection and inbreeding, as was discussed in the fall 1991 document (Part I of this two-part series). That *Bulletin* also defined basic genetic terms, introduced the concept of genetic quality control, and reviewed the role of colony management in detection and prevention of subline divergence.

This document addresses the important role of genetic monitoring, especially the monitoring of qualitative biochemical and immunological markers. Routine genetic monitoring is necessary to detect genetic contamination, the most important cause of subline divergence. Ideal markers for monitoring display simple Mendelian inheritance. They have a phenotype that is not altered by environmental factors but corresponds to the genotype. Markers should be monitored on chromosomes or linkage groups found throughout the genome. Alleles should be codominant so homozygotes and heterozygotes can be distinguished.

Skin Grafting

Skin grafting is a classical and still essential technique for characterizing inbred strains. It was developed in the 1950s by Billingham and others to detect histocompatibility differences. As histocompatibility (H) is determined by several hundred H genes found on virtually every chromosome, skin grafting reveals subline divergence due to mutation as well as to genetic contamination. Acceptance of reciprocal skin grafts, or isohistogenicity, indicates that animals are isogeneic. Isohistogenicity can therefore be used to define a strain as inbred. Various type of grafts can be performed:

autograft, when donor and recipient are the same;

- isograft or syngraft, when donor and recipient are members of the same inbred or F_1 strain;
- allograft or homograft, when donor and recipient are genetically different individuals or strains of the same species;
- xenograft or heterograft, when donor and recipient are of different species.

Grafts may be orthotopic or heterotopic, i.e., placed in a natural or unnatural position. The heterotopic graft has the advantage of being easily observed, e.g., the ear-to-body graft commonly used in the rat. The tail-to-tail graft often used in the mouse is orthotopic because the position is natural. However, to aid visibility, the graft is placed with hair growing opposite to its natural direction. To demonstrate isohistogenicity and to detect H gene mutations, skin grafting is done in the reciprocal circle pattern shown in *Figure 1.*

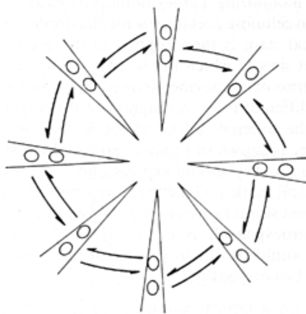


FIGURE 1. *Reciprocal circle system of skin grafting for detection of H mutations. The large Vs represent mouse tails, the ovals within the Vs represent grafts, and arrows indicate direction of grafting.*

Although skin grafting is a valuable technique, its disadvantages include a long observation time of 100 days, a large space requirement for holding animals, and a high percentage of technical failure. Moreover, isohistogenicity by itself does not indicate that either the graft donor or recipient are authentic. Graft rejection does not reveal possible sources of contamination.

The most practical way to show strain authenticity is to monitor qualitative genetic markers. These include coat color, biochemical markers that are primarily enzymes, and immunological markers such as cell surface alloantigens. In addition, advances in molecular biology have made it possible to demonstrate differences in the DNA itself, using markers known as restriction fragment length polymorphisms or RFLPs.

Monitoring of Qualitative Genetic Markers to Demonstrate Strain Authenticity

Conducting test matings to uncover hidden coat color genes. Mating can be used to determine the coat color genotype of white mice by unmasking hidden pigment genes. As explained in Part I, the allele for albinism is recessive so albino mice are c/c. The allele for albinism is also epistatic, i.e., it masks the expression of all the other coat color genes. (Non-allelic genes whose expression is masked are hypostatic.) The BALB/c mouse is homozygous agouti (A/A), but expression of the agouti phenotype is masked by the epistatic effects of the albino allele. When a BALB/c is mated to a non-agouti (a/a) C57BL/6 mouse, the F_1 offspring inherit the "C" allele for color (*Table 1*). This overrides the epistatic effect of the recessive albino "c" allele and unmasks the dominant agouti "A" allele of the BALB/c. Therefore, all offspring of a BALB/c and C57BL/6 mating should be agouti. The appearance of non-agouti offspring in the F_1 generation would show that the genotype of the putative BALB/c parent is A/a or a/a, not A/A, and therefore the BALB/c parent is genetically contaminated.

TABLE 1. *Test Mating to Unmask Hidden BALB/c Agouti Gene*

	ALLELES AT COAT COLOR LOCI				
STRAIN	**a**	**b**	**a**	**d**	**PHENOTYPE**
BALB/C	A	b	c	D	WHITE
C57L/6	a	B	C	D	BLACK
Fl HYBRID	Aa	Bb	Cc	D	BLACK AGOUTI

A/a = agouti/non agouti
B/b=black/brown
C/c=color/albino
D/d=non-dilute/dilute

Identifying biochemical markers to develop allelic profiles. The principal procedure used at Charles River is biochemical genetic monitoring. As noted above, most biochemical markers are isoenzymes. In the case of the mouse markers shown in *Table 2,* the tissues sampled are kidney and blood. The markers are polymorphic and located on chromosomes throughout the genome. To perform the

monitoring, kidney homogenates and blood are applied to cellulose acetate gels for electrophoresis. A histochemical stain is then applied to the gel, and bands appear at sites to which the isoenzymes have migrated. Allelic forms of isoenzymes, termed allozymes, migrate at slightly different rates. A diagram of the migration pattern of the different allelic forms of the dipeptidase-3 isoenzyme is shown in *Figure 2*. Since inbred strains are homozygous, they should express only one form of each biochemical marker. Genetic contamination is indicated if an inbred strain expresses the wrong form of marker or is a heterozygote. It is extremely unlikely that these changes would be due to genetic drift, i.e., mutation or residual heterozygosity.

TABLE 2. *Mouse Biochemical Genetic Markers Monitored at CRL (1/90)*

Chromosome	Gene	Description	Alleles detected
1	Idh-1	Isocitrate dehydrogenase	a,b
1	Pep-3	Dipeptidase-3	a,b
3	Car-2	Carbonic anhydrase	a,b
4	Gpd-1	Glucose-6-phosphate dehydrogenase	a,b
5	Pgm-1	Phosphoglucomutase	a,b
6	Ldr-1	Lactic dehydrogenase regulator	a,b
7	Gpi-1	Glucose phosphate isomerase	a,b
7	Hbb	Hemoglobin beta-chain	d,s
8	Es-1	Esterase-1	a,b
9	Mod-1	Malic Enzyme	a,b
11	Es-3	Esterase-3	a,b,c
14	Es-10	Esterase-10	a,b

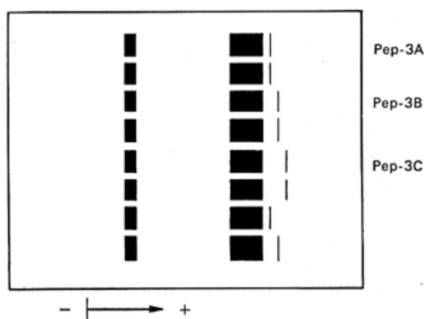


FIGURE 2. *Dipeptidase-3 Electrophoresis Gel.*

Based on a critical subset or panel of biochemical markers, an allelic profile is created to characterize an inbred strain and distinguish it from most others. For example, in *Table 3* the hemoglobin marker (Hbb) distinguishes the C57BL/6 from the other strains. Deviation from the reference profile for an inbred or F_1 hybrid strain denotes genetic contamination. Careful examination of the deviant profile may reveal the contamination source.

TABLE 3. *Allelic profiles of Charles River Inbred Mice*

			Alleles				
Marker	Chromosome	Gene	BALB/cAnN CrlBR	C3H/HeN CrlBR	C57BL/6N CrlBR	DBA/2N CrlBR	AKR/N CrlBR
Biochemical	1	Idh-1	a	a	a	b	b
	1	Pep-3	a	b	a	b	b
	3	Car-2	b	b	a	b	a
	4	Gpd-1	b	b	a	b	b

	5	Pgm-1	a	b	a	b	a
	6	Ldr-1	a	a	a	a	a
	7	Gpi-1	a	b	b	a	a
	7	Hbb	d	d	s	d	d
	8	Es-1	b	b	a	b	b
	8	Es-2	b	b	b	b	b
	9	Mod-1	a	a	b	a	b
	9	TrF	b	b	b	b	b
	11	Es-3	a	c	a	c	c
	14	Es-10	a	b	a	b	b
Immunological							
Spleen	17	H-2	d	k	b	d	k
	4	Lyb-2	b	b	b	a	c
Thymus	6	Lyt-2	b	a	b	a	a
	6	Lyt-3	b	a	b	a	a
	9	Thy-1	b	b	b	b	a
	17	Tla	+	b	b	+	b
	19	Lyt-1	b	a	b	a	b
Coat Color	2	agouti	A	A	a	a	a
	4	brown	b	B	B	b	B
	7	albino	c	C	C	C	c
	9	dilute	D	D	D	d	D

Identifying immunological markers to develop allelic profiles. Immunological markers can be divided into two groups, serum protein allotypes and cell membrane-associated or surface alloantigens. Cell surface alloantigens include major and minor histocompatibility (or transplantation) antigens that determine whether tissue grafts are accepted or rejected. A vast number of lymphocyte surface alloantigens have been described. In the mouse these include the T lymphocyte-specific Thy-1 and Lyt plus the B lymphocyte-specific Lyb antigens. RT6 and RT7 are T lymphocyte-specific alloantigens in the rat. Examples of blood group alloantigens include the Ea antigens in the mouse and RT2, 3 and 8 in the rat.

Allotypes representing two or more antigenic forms of molecules have been demonstrated in both mice and rats. These include heavy chain or Igh allotypes and kappa and lambda light chain allotypes. In addition, allelic variations of serum proteins, such as serum antigenic substance (Sas) and hemolytic complement factors (Hc) have been described in the mouse.

At Charles River, we rely mainly on methods to identify cell surface alloantigens. These are usually glycoproteins on donor tissue that are foreign to a recipient of the same species and stimulate an immune response. The response to these antigens can be mediated by both T and B lymphocytes. T lymphocyte immunity is known as cell-mediated immunity. B lymphocyte responses, which usually require help from T lymphocytes, result in the formation of antibody and are known as humoral immunity.

Cell-mediated immunity is used to characterize cell surface alloantigens by mixed lymphocyte reaction (MLR) and by skin grafting. The unidirectional MLR is used to detect major histocompatibility differences between stimulator and responder cells. *Figure 3* diagrams the major histocompatibility complexes (MHCs) of the mouse and rat, H-2 and RT1 respectively. As in other avian and mammalian species, the MHCs of rodents are highly polymorphic and consist of linked genes that code for class I and class II antigens. The class II loci are the site of the immune response genes and are the primary stimulus for MLR. In the MLR, stimulator cells (primarily B lymphocytes and macrophages) are treated to inhibit DNA synthesis with mitomycin C or gamma irradiation. They are then mixed with responder cells (T lymphocytes) in a mixed lymphocyte culture (MLC). If the appropriate antigenic differences exist between the stimulator and responder cells, the latter undergo blast transformation and proliferation, which is measured by the uptake of tritiated thymidine.

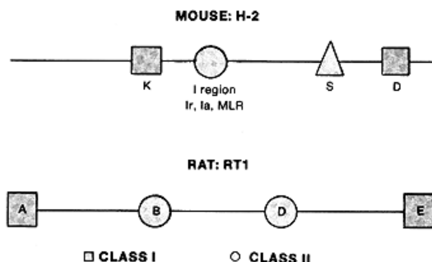


FIGURE 3. *Rodent MHC's*

Humoral immunity: Qualitative cell surface alloantigens can be identified with alloantisera or alloantibodies in cytotoxicity or hemagglutination assays. (The latter is discussed next page.) The cytotoxicity assay is based on complement-dependent cytolysis. As shown in *Figure 4,* when antibody recognizes an alloantigen on the cell surface, an antigen-antibody complex is formed. The complex fixes complement, which lyses the target cell. Cytolysis is usually demonstrated by the uptake of a vital stain.

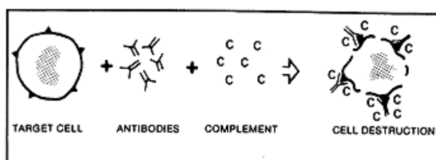


FIGURE 4. *Complement-Dependent Cytolysis.*

Table 4 lists mouse cell surface alloantigens for which we have monitored Charles River animals (see also Table 3). The markers include B and T lymphocyte alloantigens as well as the highly polymorphic H-2 complex. We use a microdroplet cytotoxicity assay much like the Terasaki method used for tissue-typing humans. Viable lymphocytes from the animals being monitored are prepared by Ficoll-Paque gradient centrifugation. These target cells are then incubated with alloantibodies. Rabbit complement is added and cytolysis, a positive reaction, is indicated by the uptake of a vital stain, eosin Y. The test is performed using microliter amounts of reagents in the Terasaki plate, which is covered with oil to prevent evaporation.

TABLE 4. Mouse Lymphocyte Surface Alloantigens Detected at CRL

	GENE	ALLELES	CHROMOSOME
SPLEEN	H-2	b,d,k,q,r	17
	Lyb-2	a,c	4
THYMUS	Thy-1	a,b	9
	Tla	a,c	17
	Lyt-1	a,b	19
	Lyt-2	a,b	6
	Lyt-3	a,b	6

Although Charles River relies principally on biochemical genetic monitoring, immunologic genetic monitoring is also used for reasons underscored by *Table 5.* In testing performed on H-2 congenic B10 strains, the biochemical allelic profiles for these strains proved identical, as expected. To distinguish the strains required

determination of their H-2 haplotypes. Both immunological and biochemical genetic monitoring yield an allelic profile that can be compared to a reference profile to determine whether a strain is authentic or contaminated, and possibly to determine the contamination source.

TABLE 5. *Genetic Profiles of H-2 Congenic B10 Strains (tested at CRL)*

MARKER	CHROM	LOCUS	C57BL/10	B10.A	B10.A(2R)	B10.02
Immunological	17	H-2 (K/D)	b/b	k/d	k/b	d/d
	17	Tla	b	b	b	b
	19	Lyt-1	b	+	b	b
	6	Lyt-2	b	b	b	b
	6	Lyt-3	b	b	b	b
	9	Thy-1	b	b	b	b
Biochemical	1	Idh-1	a	a	a	a
	1	Pep-3	a	a	a	a
	4	Gpd-1	a	a	a	a
	5	Pgm-1	a	a	a	a
	7	Gpi-1	b	b	b	b
	7	Hbb	s	s	s	s
	8	Es-1	a	a	a	a
	9	Mod-1	b	b	b	b
	11	Es-3	a	a	a	a

Table 6 lists the qualitative immunological and biochemical markers for which Charles River rats are monitored. The biochemical methods are the same as those described for mice, except gonad is collected instead of kidney. The immunological markers, which include RT1, the highly polymorphic rat MHC, are blood group antigens. They are consequently detected by hemagglutination. This positive reaction indicates that the alloantibodies have reacted with an alloantigen on the red blood cells. A negative reaction appears as the absence of hemagglutination. As in the mouse, the results of rat genetic monitoring are compiled into allelic profiles to distinguish inbred strains and thereby detect genetic contamination (*Table 7*).

TABLE 6. *Rat Genetic Markers Monitored at CRL (1/90)*

Marker	Linkage Group	Gene	Description	Alleles
Biochemical	V	Es-1	Esterase-1	a,b
	V	Es-2	Esterase-2	a,c,d
	V	Es-6	Esterase-6	a,b
	V	Es-8	Esterase-8	a,b
	V	Es-10	Esterase-10	a,b
	X	Pep-3	Peptidase	a,b
	I	Hbb	B-hemoglobin	a,b
Immunological	IX	RT1	Major histocompatibility complex	a,b,c,d f,k,l,n, u
	V	RT2	Blood group alloantigen	a,b
	-	RT3	Blood group alloantigen	a,b
	-	RT8	Blood group alloantigen	a,b

TABLE 7. *Allelic Profiles of Charles River Inbred Rats*

	Alleles

Marker	Linkage Group	Gene	F344/ CrlBR	LEW/ CrlBR	BN/ CrlBR	SHR/N CrlBR	WKY/N CrlBR
Biochemical	I	Hbb	a	b	a	a	a
	X	Pep-3	b	a	a	a	b
	V	Es-1	a	a	a	a	a
	V	Es-2	a	d	c	a	d
	V	Es-6	a	a	b	a	a
	V	Es-8	b	a	a	b	a
	V	Es-10	a	b	b	a	b
	II	Mup-1	b	b	a	a	a
Blood Group Alloantigen	IX	RT1	l^v	l	n	k	l
	V	RT2	a	a	a	b	a
	--	RT3	b	a	b	a	a
	--	RT8	b	b	b	a	b

Note: Techniques used by Charles River for routine genetic monitoring of its inbred and F_1 hybrid strains have also been used to characterize the allelic frequencies in its outbred stocks. *Table 8* shows the prevalence of various RT1 haplotypes in the Crl:CD®BR Sprague-Dawley rats from our colonies. As expected, since the CD rat is outbred, more than one RT1 haplotype was identified; most common is u, followed by l and b. Almost all CD rats, irrespective of source colony, are $RT2^b$, $RT3^a$ and $RT8^b$. In summary, monitoring of outbred stocks found the same haplotypes and alleles represented, at similar frequencies, in all CD colonies.

TABLE 8. *RT1 Haplotype and Blood Group Allele Frequencies in Crl: CD® (SD) BR Rats*

A. Major Histocompatibility Complex (RT1)					
		RT1 Haplotype Frequencies (%)			
Facility-Area	No.Tested	b/f	d	l	u
WIL	30	10.0	--	6.7	83.3
CRK	40	18.8	--	15.0	66.2
CRC	36	9.7	4.2	23.6	62.5
CRW-1	20	25.0	2.5	5.0	67.5
CRW-8	20	15.0	2.5	15.0	67.5
CRJ-11	40	15.0	--	15.0	70.0
CRJ-21	40	20.0	6.3	27.5	46.2
CRUK-51	40	16.3	2.5	38.7	42.5
CRUK-56	40	8.3	8.8	38.0	45.0

B. Erythrocyte Alloantigens							
		Allelic Frequencies(%)					
		RT2		RT3		RT8	
Facility-Area	No.Tested	a	b	a,a/b	b/b	a	b
WIL	30	6.7	93.3	100	--	--	100
CRK	40	1.3	98.3	100	--	--	100
CRC	36	--	100	100	--	--	100
CRW-1	20	--	100	90	10	--	100
CRW-8	20	--	100	95	5	--	100
CRJ-11	40	1.2	98.8	100	--	--	100
CRJ-21	40	1.2	98.8	100	--	--	100
CRUK-51	40	--	100	100	--	--	100
CRUK-56	40	--	100	98	2	--	100

WIL=Wilmington facility; CRK=Kingston; CRC=Canada;

CRW=Wiga (German); CRJ=Japan; CRUK=United Kingdom

Detecting restriction fragment length polymorphisms. Detection of RFLPs (commonly known as "riflips") has been made possible by discovery of restriction enzymes. Also called restriction endonucleases, these are found in a wide variety of bacteria. They recognize specific sequences of 4 to 8 base pairs in double-stranded DNA and cleave both strands of the duplex at specific sites. RFLPs occur when two individuals differ in the presence or absence of these restriction sites in their DNA, or by insertion or deletion of DNA between two sites. RFLPs abound in all populations of plants and animals studied to date. Individuals within an inbred strain are generally expected to have identical restriction sites because they are isogeneic.

RFLPs are detected by a hybridization procedure known as Southern Blotting because it was devised by the scientist E.M. Southern (*Figure 5*). Once exposed to a restriction enzyme, DNA fragments are separated according to their length by agarose-gel electrophoresis. The separated fragments are blotted onto a sheet of nitrocellulose or other material. In the example shown, the fragment or fragments containing the target DNA are localized by hybridization with a ^{32}P-1abeled DNA probe, followed by autoradiography. (When RNA rather than DNA is separated by electrophoresis, the procedure is called "Northern blotting." When proteins are separated by electrophoresis, blotted onto nitrocellulose and identified with specific antibodies, it is "Western blotting.")

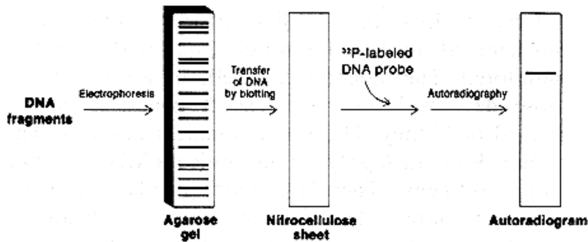
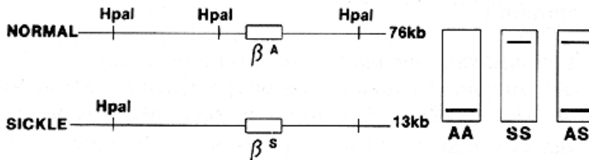


FIGURE 5. *Southern blotting.*

An example of a betaglobin RFLP associated with sickle cell anemia in humans is shown in *Table 9,*. Unlike normal individuals, those with this disease lack an Hpal restriction site. Consequently, the restriction enzyme fragment containing the betaglobin gene in people with sickle cell anemia (SS) is larger than in normals (AA). Both the large and small fragments are present in the AS heterozygote.

TABLE 9. *A Betaglobin RFLP Associated with Sickle Cell Anemia*



RFLP analysis has been used by Ted Kurtz and colleagues at the University of California at San Francisco to characterize SHR and WKY rat strains (*Hypertension* 10:127, 1987; 13:189, 1989) and distinguish between inbred mouse strains. Their probe is an alkaline-phosphatase conjugated oligonucleotide that detects loci scattered throughout the genome, rather than a single locus. When a blot of rodent DNA is hybridized with this probe and incubated with an alkaline-phosphatase substrate, a DNA fingerprint with numerous bands appears. The fingerprint patterns are different for genetically different individuals and strains. Using this DNA

fingerprinting technique, Kurtz et al. demonstrated that while SHR rats from different vendors are inbred and genetically uniform, WKY rats from different sources have DNA fingerprints that differ from one another and from the SHR strain. WKY rats from one vendor were found not to be inbred.

The Charles River Genetic Monitoring Program

The mainstay of the CRL program is biochemical monitoring, with a focus on 9 markers in the mouse and 7 markers in the rat. These critical subsets were chosen because they distinguish among all our rodent strains.

Inbred colonies are monitored twice yearly. F_1 hybrid colonies are monitored quarterly because they are more subject to genetic contamination, as F_1 hybrid mice may have the same coat color as one of the parental strains. For example, the B6C3F_1 hybrid has a dark agouti coat color like the C3H parental strain.

Five nucleus animals and fifteen expansion or production animals are tested from each colony. When the expansion colony is lacking or too small to provide fifteen animals, the production colony is sampled. As a general rule, sampling high in the colony pyramid (i.e., from the nucleus and expansion colonies) provides more information than testing the production colony. Sample size at Charles River is based on experience and economic considerations, not on a statistical formula.

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