Int J Legal Med (2005) 119: 295–302
DOI 10.1007/s00414-005-0545-9

TECHNICAL NOTE

Bruce Budowle · Paolo Garofano · Andreas Hellman · Melba Ketchum · Sree Kanthaswamy · Walther Parson · Wim van Haeringen · Steve Fain · Tom Broad

Recommendations for animal DNA forensic and identity testing

Received: 29 November 2004 / Accepted: 29 March 2005 / Published online: 16 April 2005

Abstract Genetic analysis in animals has been used for many applications, such as kinship analysis, for determining the sire of an offspring when a female has been exposed to multiple males, determining parentage when an animal switches offspring with another dam, extended lineage reconstruction, estimating inbreeding, identification in breed registries, and speciation. It now also is being used increasingly to characterize animal materials in forensic cases. As such, it is important to operate under a set of minimum guidelines that assures that all service providers have a template to follow for quality practices. None have been delineated for animal genetic identity testing. Based on the model for human DNA forensic analyses, a basic discussion of the issues and guidelines is provided for animal testing to include analytical practices, data evaluation, nomenclature, allele designation, statistics, validation, proficiency testing, lineage markers, casework files, and reporting. These should provide a basis for professional societies and/or working groups to establish more formalized recommendations.

Keywords Forensic science · Guidelines · Animal genetics · DNA typing

B. Budowle (✉)
FBI Laboratory,
2501 Investigation Parkway,
Quantico, VA 22135, USA
e-mail: bruce.budowle@ic.fbi.gov
Tel.: +1-703-6328386
Fax: +1-703-6327817

P. Garofano
Forensic Genetics Division
UNIRELAB s.r.l., Milan, 20019, Italy

A. Hellman
Bundeskriminalamt Kriminaltechnisches Institut,
KT32, 65173 Wiesbaden, Germany

M. Ketchum
DNA Diagnostics dba Shelterwood Laboratories,
Timpson, TX 75975, USA

S. Kanthaswamy
Veterinary Genetics Laboratory-Forensics,
School of Veterinary Medicine,
University of California,
Davis, CA 95616, USA

W. Parson
Institute of Legal Medicine,
Innsbruck Medical University,
6020 Innsbruck, Austria

W. van Haeringen
Dr. Van Haeringen Laboratorium BV,
6708 Wageningen, The Netherlands

S. Fain
National Forensics Laboratory,
U.S. Fish and Wildlife Service,
Ashland, OR 97520, USA

T. Broad
Australian Equine Genetics Research Centre,
University of Queensland,
St Lucia, QLD 4072, Australia

Introduction

Animal DNA forensic and identity testing is rapidly becoming commonplace such as in resolving criminal and civil cases, kinship analysis, determining the sire of an offspring when a female has been exposed to multiple males, determining parentage when an animal switches offspring with another dam, extended lineage reconstruction, estimating inbreeding, identification in breed registries, and speciation, to name a few [1–16]. Almost any species to which an animal belongs can be genetically characterized to a high degree of certainty. Domestic animals, agriculturally important animals, and selected wildlife species have been the focus; these include bears, birds, cats, deer, dogs, wolves, fish, sheep, goats, cattle, and horses. As the demand for animal forensic and identity genetic testing grows, it has become increasingly important to have a set of minimum guidelines that assures that all service providers have a template to follow for quality practices that can

withstand legal scrutiny. Quality assurance (QA) and quality control (QC) are requisite for ensuring good laboratory practices and high-quality results. QA and QC are not synonymous. QA is the monitoring of activities that are intended to verify whether practices and test results are providing reliable information. QC is a mechanism or activity(ies) intended to verify whether test conditions are functioning appropriately to yield defined accurate and reproducible results.

The QA and QC measures for DNA typing are well established in human forensic and paternity testing arenas [17–22]. Therefore, many of the recommendations for animal identity testing can be based on that wealth of experience and need not be reinvented; some, however, may require slight modifications particularly because some of the methods employed may be breed- or species-specific. For example, those animal testing laboratories using methods that are species-specific do not need to be concerned with cross-contamination of test samples with DNA inadvertently shed from the staff, as do human identity testing laboratories. Therefore, there is no need to keep on file the profiles of the scientists and technicians. Importantly, the recommendations herein should not be construed as questioning the reliability of genetic identity testing of animal tissues. The general procedures used today are reliable. However, there has not been a peer consensus process (until now) to establish a baseline for laboratories to structure their QA practices and to build some accepted uniformity of quality practices among laboratories. The obvious benefits are as follows: (1) a minimum performance baseline allows contractors, such as a registry, or the public of what might occur when a civil or criminal case is investigated or a government agency protecting wildlife resources to have confidence in the results from individual service providers; (2) the customer can have a list of criteria to assess a provider before requesting a service; and (3) the service providers can be confident that when they operate within the recommended guidelines, they will be meeting independently established systems of best practice and that their results will be less open to challenge.

Basic analytical practices

To obtain reliable results, basic analytical equipment should be employed and standard operating protocols (SOPs) should be established. Essential equipment includes biosafety hoods, refrigerators/freezers, thermal cyclers, calibrated pipettes, and an electrophoretic platform capable of resolving DNA fragments differing in length by one base (the latter applies to those laboratories typing microsatellites or performing sequencing). SOPs are detailed recipes describing the entire process of evidence handling and analysis from sample collection and transfer to the laboratory (if appropriate) to interpretation of analytical results and preparation of the report, all being well documented. Because most molecular biology assays are PCR-based, it is important to design the process with the prospect that contamination may arise. The accepted practice is to physically separate pre-PCR and post-PCR operations. All initial sample manipulations, such as DNA extraction, and PCR setup steps must be performed in the laboratory where no amplified DNA is handled or stored. Containment hoods, reagents, and pipettes should be dedicated to the pre-PCR and post-PCR area.

Incidents of contamination can be reduced further by using dedicated lab coats, disposable gloves, and, particularly for forensic use, aerosol-resistant pipette tips. When possible, items of forensic evidence should be extracted and amplified prior to known reference samples. Work surface areas should be thoroughly cleaned with freshly prepared 10% bleach on a regular basis. Dependent upon the type of analysis, workspaces under dedicated hoods should be employed. Pipettes and workstations under dedicated hoods should be cleaned and exposed to ultraviolet light, as necessary. In addition, autoclaving and/or exposing to UV light all appropriate materials and reagents should be carried out.

It is important that the analysis itself be monitored to demonstrate that the results obtained are reliable. Such QC requires that certain practices be carried out. These include the following: reagents critical to the procedure should be tested before analyzing unknown samples; essential equipment should be calibrated on a regular basis (at least based on the manufacturer's recommendations or more often if needed); and known positive and negative control samples should be used with the PCR analysis. The increased sensitivity of the PCR requires control measure monitoring for detecting levels of contamination that may affect the interpretation of an assay. Negative controls are useful for monitoring general contamination of the reagents and materials used in preparing samples and during the PCR. Each sample or set of samples should include a reagent blank in the PCR analysis. The reagent blank consists of all reagents used in the analytical process, except that no template DNA is added, and is processed through the entire extraction, amplification, and subsequent typing procedures. If more than one type of extraction procedure is employed (with different extraction reagents), then a reagent blank is set up for each type of extraction or group of extraction reagents used. A negative control is set up at the amplification stage. The negative control contains all reagents required for the PCR, except that no template DNA is added. It is processed through the PCR and typing procedures. Purified water is placed in the reagent and negative controls in lieu of template DNA in a volume equal to what would be used if DNA were introduced. A positive control, which is a sample of known type, should be run with each sample or set of samples processed. The positive control's DNA profile should display the correct type or the results of the particular analysis set are invalid. A positive control can be any verified/characterized DNA sample. However, a cell line DNA that is universally accessible would be desirable. This positive control would allow for more effective interlaboratory comparison as well as monitor intralaboratory performance over time. If gender determination is the primary objective of the analysis (e.g., sex typing of birds), then male and female positive

controls should be used. Similarly, if gene tests (for genetic disorders, coat color, etc.) are performed, positive (homozygous and heterozygous) and negative controls should also be used.

With the increased sensitivity afforded by PCR procedures and particularly when typing very low quantity DNA samples, practices to minimize contamination are unlikely to eliminate contamination entirely. Therefore, a contamination log should be maintained. The log should contain a sufficient description of the contamination and where it arose, what measures were taken to identify and mollify the contamination, and genetic profiles of the contaminant. Such information can be invaluable in identifying and reducing laboratory-wide or interlaboratory-wide contamination sources.

With advances in robotics, it is anticipated that both pre-PCR and post-PCR operations may be accommodated within one robotic system. Such a design does not adhere to the separation of operations recommendation, but yet may improve the analytical process. Progress should not be stifled; therefore, implementation of such a robotic system requires demonstrating that contamination is no greater than what occurs with a conventional separate pre-PCR and post-PCR operation.

Data evaluation

The SOP should contain a description of the criteria to be used to assess a genetic profile. These include identifying an allele (such as a peak or band for a STR allele), accepting an allele as typeable (such as off-scale or low-level data assessments), addressing artifacts, addressing mixtures, and addressing contamination (such as reanalysis). Currently, dealing with samples that may be mixtures of tissues from several individuals, or that may be obtainable in suboptimal amounts, is atypical of the routine typing experience of most animal service typing laboratories. But, for forensic applications, these possibilities should be in the mindset of the service laboratory and its typing procedures, and interpretation guidelines should address the occurrence of mixtures, when applicable. Routine service animal genotyping labs, such as registries, usually do not address analysis of mixtures and are typically not limited by sample quantity, which forensic laboratories often must consider. Therefore, some service providers may suggest that many of the QA and QC procedures that are essential for forensic applications have no relevance for their labs. We strongly counsel against this view. Making assumptions about sample integrity and quality is at best unwarranted and could be counterproductive. Moreover, investments in quality practices improve performance, increase confidence, and promote innovation.

Nomenclature

Standardized or common nomenclature for loci promotes exchange and comparison of data within and among laboratories. The nomenclature advocated for human DNA markers is an effective model [17]. DNA sequences are read in the 5′–3′direction. However, for STR loci, the choice of the strand of the DNA duplex molecule that is read can influence the repeat motif designation. For protein-coding genes and STR sequences in the intron of a gene, the encoding strand is used to characterize the repeat motif of the alleles. For repetitive sequences of anonymous STR loci, the sequence first described in the literature or the first public database entry should be the basis for nomenclature. The repeat sequence motif should be designated on the first 5′ nucleotide that can define a repeat motif. If at all possible, a standardized nomenclature should be instituted. The D#S# or DYS# nomenclature should be considered for naming loci [4]. Adopting this or a similar system conveys whether or not the locus has been mapped and on which chromosome it resides. There are currently many STR loci that are widely used for animal identity testing but with nomenclature that would not adhere to these recommendations. Although it would be desirable to convert the names to these recommendations in those species for which the map locations of marker loci have been determined, to avoid confusion, the ad hoc nomenclature should continue being used.

Allele designation

Allele designation systems used in animal identity testing are by letter, amplicon size, or repeat unit. Under the letter designation system, fragment length is converted to a letter name based on the distribution of alleles. Consider horse breed A is typed for locus L and 11 alleles are discovered in a sample population study of 100 individuals. For this example, all alleles increase by two bases (i.e., a dinucleotide repeat system) from the smallest allele 1 to the largest allele 11. The middle allele in the observed distribution, in this case allele 6, is designated as "M." Alleles 5, 4, 3, 2, and 1 are designated L, K, J, I, and H, respectively, and alleles 7, 8, 9, 10, and 11 are designated N, O, P, Q, and R, respectively. This could be an unwieldy nomenclature because it does not allow for effective interlaboratory comparison. With domestic and agricultural animals, there is strong manmade selective pressure. It is entirely possible that horse breed B could also yield 11 alleles, but the distribution is different than breed A. If breed B's alleles were equivalent to breed A's alleles 2–12, this would not be apparent when comparing or compiling data. The M allele of breed B would be equivalent to the N allele of breed A. Moreover, the letter system is not well designed for a locus with more than 26 alleles.

The amplicon allele nomenclature is based on base length estimated by electrophoretic migration. The mass assessment of an allele is not necessarily accurate, although it can be precise if electrophoretic conditions are the same. The outcome is that the estimated number of bases comprising an amplicon and used to name the allele may not be correct but can be reproducible. Moreover, the same fragments can differ up to a few bases in number when different

electrophoretic systems are employed [23]. Thus, direct comparison between laboratories of base number, as well as allelic letter designations, requires that these data be normalized or standardized. This phenomenon does not call into question the reliability of typing within a laboratory; it just makes interlaboratory comparison complicated. There is quite a bit of crossbreeding between breeds within domestic species, and when the progenies of these interbreed crosses have been typed, their alleles typically have consistent assignments. Therefore, it can be argued that it is a reasonable assumption that, for example, the L allele of locus ASB2 in trotters is the same as that in Arabians. The concern, though, is ensuring that correct allele assignments are made among all laboratories and providing a minimum level of confidence in the legal setting.

A system that allows for the unequivocal exchange of genotype information between laboratories is allele designation based operationally on the number of repeat sequences contained within the amplicon. The designation of alleles that contain a partial repeat motif (i.e., a repeat sequence that is smaller in size than the general repeat size for the specified locus) can be designated based on the number of complete repeats and, separated by a point, the number of bases of the incomplete repeat. An example would be an allele of a tetranucleotide repeat that contains eight full repeats and a partial repeat of only two bases. It would be designated as an 8.2. Another example would be an allele, comprised of dinucleotide repeats, containing five repeat motifs and a partial repeat (one extra base); it would be designated as 5.1. It is important that the repeat motif of the locus be well defined so the partial repeat nomenclature is compatible. For example, a tetranucleotide repeat locus can have X, X.1, X.2, and X.3 alleles, whereas a dinucleotide repeat locus can have only X and X.1 alleles.

Alleles named by these different designation systems can be normalized by exchange of well-characterized cell lines or reference DNA samples. For some species, cell lines may be available as controls whose alleles can be sequenced for the loci of interest and should be considered. An effective approach for standardizing allele designation data is the implementation of allelic ladders. An allelic ladder contains the common alleles of a STR locus and is used as a reference for allele designation (in concept, this is no different than typing a bi-allelic SNP where a sample with both alleles is typed as a control). The general spacing of the rungs of a ladder is based on the basic repeat motif of a STR system (although common partial repeat alleles can also be included in the ladder). The alleles contained within the ladder should be sequenced, so there is no question about the repeat number contained within each allele of the ladder. The alleles of unknown samples are designated operationally by comparison within an allelic ladder (and internal size standards for fluorescent-based detection systems). Although electrophoretic conditions can affect mobility, and hence size determination of an allele, the alleles in an allelic ladder are similar in sequence to those in the sample and thus mobility should be similar. Different laboratories may use different samples to construct an allelic ladder(s). However, interlaboratory comparisons can still be made because all ladder alleles have been sequenced. Allelic ladder data should be made publicly available.

Statistics

When interpreting forensic evidence or evaluating a kinship analysis, a qualitative and quantitative statement about the outcome of the analysis should be provided [24]. The general approaches to these statements should be contained in the interpretation section of the SOP.

Population data are required to estimate the frequency of alleles for each locus. The reference databases typically are comprised of samples of “unrelated” individuals that are conveniently acquired. Because inferences of rarity are based on the sample population analyzed and assumptions of relevance and representativeness are basic to identity testing, the reference population data used should be cited. The reference database needs to be defined with reference to how it was constructed. For example, dogs are not as mobile as their human counterparts and only a small percentage of dogs have offspring. In addition, veterinarians may describe a dog’s breed by the predominant breed features, even if there is evidence of a mixture. Thus, the assumptions of the database need to be disclosed. One can make assumptions on the estimates of inbreeding. However, access to population data can provide empirical information on the degree of inbreeding to effect better statistical estimates. The population data (i.e., the DNA profiles) should be made available upon request for review.

When a comparison of DNA profiles derived from unknown and reference samples fails to exclude an individual as a contributor of the evidence sample or as biologically related, a statistical assessment and/or probabilistic reasoning are used to convey the significance of the finding [24]. For animal genetics identity testing, the statistical conclusions should be conveyed appropriately. These recommendations herein do not address the best statistical method to use because there are a number of different methods for estimating the rarity of the evidence, including counting, random match probabilities, likelihood ratios, probability of exclusion, and Bayesian methods [24–26]. Depending on the application and if used appropriately, any of these statistical approaches can be considered to draw valid conclusions. So that statistical method(s) used in an analysis can be assessed, the SOP should contain a section describing the statistical methods used, including the algorithm and reference citations.

For kinship analyses, mutational events must be considered. This is particularly so for STR loci which have relatively high mutation rates [27, 28]. Statistical methods for kinship (and lineage studies) should incorporate a mechanism for assessing mutations. To better assess the mutation rate per locus, mutations, when observed, should be documented. Data collection should include whether

the mutation is paternal or maternal in origin (or inconclusive), the total number of meioses analyzed (no mutations and mutations), the allele(s) that mutated, the type of mutation (repeat gain or loss, null allele, duplication, transition, transversion), and, if a repeat change, the number of repeats (as an increase or decrease in size).

Collecting (geographic and breed-specific) population and mutation data can be facilitated by collaborative efforts. The community should develop a central repository for population and mutation data so all can benefit and a more sound foundation of population genetics can be enjoyed. One approach to generate substantial data is to establish a minimum set of core loci.

Methodology validation

All protocols used by the laboratory should be validated before their use for casework. Validation is a process by which a procedure is evaluated to determine its efficacy and reliability and to determine the operational limits of the technique. The studies include, when appropriate, sensitivity, specificity, reproducibility, precision, accuracy, testing the parameters of a method, and analyzing samples (mock or nonprobative) commensurate with the intentions for use of the assay. Appropriate literature references that support the fundamentals or establish the validity of a method should be documented.

Material or significant revisions on existing protocols should undergo validation commensurate with the modification. The revised protocol should be documented, dated, and identified as a more recent version. Already validated protocols obtained from other laboratories should still undergo an internal validation prior to their use on casework. Before beginning routine casework, scientists and/or other practitioners should successfully complete a qualifying test using the procedure.

Proficiency testing

Participation in a proficiency testing program is an essential element of a successful QA/QC program. Proficiency testing is used to periodically demonstrate the quality performance of the DNA typing laboratory and serves as a mechanism for critical self-evaluation. In addition, the process of using a uniform proficiency testing program ensures that results are correct and sets standards of performance. The program tests the ability of the laboratory to produce acceptable results, and it motivates, in a professional peer-review manner, laboratories to comply with accepted state-of-the-art performance. Ancillary benefits of a proficiency testing program are standards/standardization of methods and procedures, standardization of nomenclature, evaluation of competency, and reduction in errors.

The laboratory should participate in proficiency testing conducted by an outside agency, if possible, and the tests should be appropriately designed. If no outside agency provides an appropriate test, then an exchange program with properly designed tests can be implemented between or among laboratories, or even within a laboratory if the functions of test provider and test taker are separated and documented. One mechanism to accomplish proficiency testing is by analysis and reporting of results from appropriate biological specimens submitted to the laboratory as an open test. With an open proficiency test, the laboratory and its staff are aware that they are being tested but are not aware of the DNA typing results; they are blind regarding the results. Specimens are analyzed and interpreted according to the protocol approved by the laboratory. Open proficiency testing should be performed at least once a year.

Because some animal genetic identity testing services employ high throughput methods, an additional QA practice could be for the contractor to send a subset of duplicate samples. These samples would be labeled such that they would not be determined to be duplicates and would be completely blind to the laboratory. However, this practice would require quality control and sufficient documentation by the sample provider to ensure that samples are not mixed up prior to submission to the laboratory. Unless such practices are in place, retesting a subset of samples should not be considered.

A proficiency testing program alone is not sufficient; laboratories need to have and maintain proper documentation and, if errors occur, have in place policies and practices for corrective action. There are different types of error that can occur:

- Administrative error—any discrepancy in a proficiency test determined to be the result of an administrative error (i.e., clerical, sample mix-up, improper storage, documentation, etc.) may require consultation with the analyst or person/organization submitting the sample.
- Systematic error—any discrepancy in a proficiency test determined to be the result of a systematic error (i.e., equipment, materials, environment, etc.) may require a review of all relevant work/database results around the time of the test back to the last proficiency test. Once the cause of the discrepancy is identified and corrective action is taken, an education process should take place.
- Analytical/interpretative error—any discrepancy in a proficiency test determined to be the result of an analytical/interpretative error (e.g., false match) should prohibit further typing until the problem is identified and corrected. An additional set of proficiency samples should be analyzed if an analytical/interpretative error occurs.

Lineage markers

Some genetic markers are inherited uniparentally such as mitochondrial DNA (mtDNA) and markers residing on nonrecombining regions of gender determining sex chromosomes, such as the Y-chromosome of mammals and the

Z-chromosome of birds and amphibians. MtDNA can be analyzed by sequencing, SNP assays, or restriction digestion typing and has been used widely in animal typing. Y-linked variation is assessed at polymorphic STR, VNTR, and SNP loci but has only more recently been applied to animal typing. The QA/QC practices for autosomal loci described above also apply for these mitochondrial and Y-chromosome lineage markers. However, there are some additional suggestions that apply to lineage-based systems.

MtDNA typing in animals has primarily been used for species identification, population assignment, verification of food products, identification of animal fibers in clothing, and identification in poaching cases, to name a few applications, and has focused primarily on the cytochrome *b* oxidase gene [29–36]. However, mtDNA analyses (including the cytochrome *b* oxidase gene and the hypervariable noncoding region) also are increasingly being used in criminal cases primarily to characterize animal hairs (primarily dog and cat) found at crime scenes. Thus, stricter guidelines for forensic use are warranted. Nomenclature for mtDNA variants is different than that used for STR loci. A sequence from a sample should be aligned with a reference sequence, and only sites that differ from the reference should be scored [18]. As an example, consider that for the domestic dog at nucleotide position 100, the reference sequence has an A and the unknown sample exhibits a G. The sample's mtDNA type should be listed as 100G, and, since no other sites are listed, they are considered identical to the reference sequence. An insertion immediately subsequent to a site should be designated as the site position followed by a point and the number 1, 2, and so on depending on the number of insertions. For example, if an insertion of a C were to occur immediately after site 100, then it would be designated as 100.1 C. Similarly, for a deletion at site 100, it would be designated 100−. All nomenclature recommendations are intended to be compatible with IUPAC codes. For more details on nomenclature, see [18].

Reference mtDNA genome sequences are available for the domestic species cat (NC_001700), chicken (NC_001323), cow (NC_001567), dog (NC_002008), goat (NC_005044), horse (NC_001640), pig (NC_000845), and sheep (NC_001941) and for wildlife species of deer (NC_004563), bear (NC_003426), salmon (NC_001960), and sturgeon (NC_004420). Thus, for most relevant domestic and wildlife species, reference mtDNA templates can be established, and all sites were given a location number to facilitate nomenclature.

Y-marker typing has been used for identifying paternal lineages in some animals [37–44]. Because Y-like systems are haploid, they typically display only one allele per locus. Sometimes, more than one allele is observed (due to gene duplication, translocation, gene conversion, etc.). To convey the duplication, a hyphen should be used between the alleles. For example, consider the designation of a type as 11–13. For more details, see [20].

For assessing statistical significance of markers that reside in nonrecombinant regions, it is not valid to assume independence and multiply allele frequencies of the variant sites or loci in a sample profile. The statistical method used in assessing the weight of lineage-based markers should be described in an SOP, including the algorithm and reference citations.

Forensic casework files

Casework dossiers should be initiated for each forensic case. Upon completion of a case, all original paperwork, lab notes, analysis notes, internal chain of custody documentation, photographs, photocopies of evidence packets, and a copy of the report should be placed into the casework file with proper pagination. Each case should undergo a technical and administrative review by qualified practitioner(s), and the results from those reviews should be included in the casework file. When insufficiencies or discrepancies are noted, they should be rectified before completion and final approval (signing) of the case.

Final reporting

A brief concisely written final report should be prepared for the client that summarizes the analytical results, interpretation, and conclusions based on the analytical data. If warranted, the conclusions should clearly state appropriate qualifications or limitations on the evidence interpretation. Example reports can be obtained upon request.

Conclusions

This is the first set of guidelines for animal genetic identity testing to be published. They should not be considered as set in stone, but instead as dynamic and as an initial foundation for consideration. We realize that it will take time and resources to implement these recommendations, and they cannot be instituted immediately. However, we do recommend that the community move in this direction with reasonable speed to ensure that practitioners have quality systems in place. We also recognize that the nonforensic animal identity market may not be willing or able to bear the costs to adopt all the QA practices recommended herein. If so, then: (1) for any protocol or equipment utilized for nonforensic identity applications that falls short of the minimum guidelines, validation data demonstrating that the results from the assay are reliable should be maintained on file and provided upon request; or (2) those recommended QA practices that are not part of the service should be disclosed to the customer. Alternate approaches that meet the spirit of the recommendations obviously should be considered as viable mechanisms for establishing quality practices. Also, standards such as the specific ISO/IEC 17025:1999 *General requirements for the competence of testing and calibration laboratories* (that apply to all service genotyping providers) or the generic International Organization for Standardization (ISO), International Laboratory Accreditation Cooperation (ILAC), Asia Pacific

Laboratory Accreditation Cooperation (APLAC), or OECD Panel on Good Laboratory Practice (GLP) could be consulted to augment these guidelines. These guidelines reported herein are being provided so that the relevant community (professional societies and/or working groups) can assess them, consider best practices, and make recommendations that can better effect basic practices.

Acknowledgements We thank the International Society for Animal Genetics and the Japan Organizing Committee of the 9th International Conference on Animal Genetics held in Tokyo, Japan, on 12–16 September 2004, for providing a workshop forum for the discussions that led to the drafting of these guidelines. Presentations made at the Animal Forensics Workshop in Tokyo can be viewed at http://www.vgl.ucdavis.edu/forensics/ISAG/index.html.

This is publication number 05-04 of the Laboratory Division of the Federal Bureau of Investigation. Names of commercial manufacturers are provided for identification only, and inclusion does not imply endorsement by the Federal Bureau of Investigation.

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