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**Wildlife Forensics Validation Standard—Validating New  
Primers for Sequencing**



## Wildlife Forensics Validation Standard—Validating New Primers for Sequencing

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## Foreword

This validation document addresses the particular need in wildlife forensics to validate new primer sets for sequencing applications. It is intended to accommodate the diversity of species and substrates encountered in wildlife forensic laboratories that have previously validated a DNA sequencing method and are developing new primers to be used with that method.

This standard has been revised, prepared, and finalized by the Wildlife Forensics Consensus Body of the AAFS Standards Board. It was developed in the OSAC Wildlife Subcommittee Validation Task Group, reviewed by the OSAC Wildlife Subcommittee and presented to the Biology Subject Area Committee for movement through the AAFS Standards Board. All hyperlinks and web addresses shown in this document are current as the publication date of this standard.

**Keywords:** *wildlife forensics, validation, DNA sequencing, mitochondria, chromosome*

**Abstract:** This document provides minimum standards and recommendations for validating new primers for mitochondrial haplotyping and/or taxonomic identification via sequencing in wildlife forensic DNA laboratories where the sequencing (Sanger) method has already been validated.

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# Wildlife Forensics Validation Standard—Validating New Primers for Sequencing

## 1 Scope

This document provides minimum requirements and recommendations for validating new primers for mitochondrial haplotyping and/or taxonomic identification via sequencing in wildlife forensic DNA laboratories where the sequencing (Sanger) method has already been validated.

## 2 Normative References

There are no normative reference documents. Annex C, Bibliography, contains informative references.

## 3 Terms and Definitions

For purposes of this document, the following definitions apply.

### 3.1

#### **amplicon**

Any PCR amplification product.

### 3.2

#### **amplification**

An increase in the number of copies of a specific DNA fragment; can be in vivo or in vitro. In forensic DNA testing laboratories, this refers to the use of the PCR technique to produce many more copies of DNA at specific genetic loci.

### 3.3

#### **capillary electrophoresis**

An electrophoretic technique for separating DNA molecules by their relative size based on their differential migration through a capillary tube filled with a liquid polymer.

### 3.4

#### **case-type samples**

Samples from known individuals with known testing results prepared within the laboratory to simulate a range of samples typically encountered by the testing laboratory in casework. The use of a range of test samples in validation studies facilitates the development of protocols for casework.

### 3.5

#### **critical reagents**

Chemicals or other materials used in testing whose performance is vital to the success of the test as determined by empirical studies or routine practice.

### 3.6

#### **genus**

A level of taxonomic classification that defines a group of related species.

**3.7****haplotype**

A set of linked DNA variations, or polymorphisms, that tend to be inherited together (e.g. commonly used for mitochondrial or Y-chromosome analysis). A haplotype can refer to a combination of alleles or to a set of single nucleotide polymorphisms (SNPs) found on the same chromosome.

**3.8****heteroplasmy**

The presence of more than one mitochondrial DNA (mtDNA) sequence or type within a single individual.

**3.9****marker (DNA)**

A gene or specific DNA sequence of known location on a chromosome or genome.

**3.10****Mitochondrial DNA****mtDNA**

A small, circular DNA molecule located in eukaryotic mitochondria that is typically maternally inherited; the resistance to degradation and, presence of multiple copies of mtDNA in each cell makes it useful with samples originating from limited or damaged biological material.

**3.11****phylogenetic coverage**

A criterion used to guide the selection of species for comparative analysis and species determination; the selection of species is based on genetic relatedness.

**3.12****polymerase chain reaction****PCR**

An enzymatic process by which a specific region of DNA is replicated during repetitive cycles that consist of the following: denaturation of the template; annealing of primers to complementary sequences at an empirically determined temperature; and extension of the bound primers by a DNA polymerase. The goal of the PCR process is to generate many copies (termed products or amplicons) of a specific region of DNA for further analysis.

**3.13****polymerase**

An enzyme that catalyzes the synthesis of nucleic acids on preexisting nucleic acid templates.

**3.14****polymorphism**

The occurrence in a population of two or more alleles or physical traits at a genetic locus. This variation within a population permits the differentiation of individuals via DNA testing or physical traits.

**3.15****population**

A group of organisms of the same species in a defined geographic area, such that any pair of members can interbreed.

**3.16****primer**

A short polynucleotide chain, usually 18–30 bases long, which targets a specific region of the template DNA and allows a DNA polymerase to initiate synthesis of a complementary strand; two primers are required for amplification of a particular section of double-stranded DNA. The primers are complementary to opposite strands and are designed to bracket the region of interest for amplification. Polymerization is initiated at the 3' end of the primer and extends in a 5' to 3' manner.

**3.17****reference materials**

Biological specimens of known identity or data derived from them.

**3.18****Sanger sequencing**

A method of DNA sequencing for determining the order of bases in a DNA molecule based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. See sequencing below.

**3.19****sensitivity studies**

A set of critical studies performed during developmental and/or internal validation of DNA or other test methods designed to define the lower and upper limits/ bounds of an assay to accurately detect an analyte.

**3.20****sequencing (DNA)**

A laboratory technique used to determine the sequence of bases (A, C, G, and T) in a DNA molecule.

**3.21****species**

The fundamental unit of taxonomic classification. There is no singular species definition in biology. Essentially the term denotes a group of organisms with a unique shared evolutionary lineage.

**3.22****species specificity**

A reference to studies generally performed during developmental validation of forensic testing assays to assess if non-targeted species are detected in an assay (e.g., detection of microbial DNA in a human assay). The detection of non-targeted species does not necessarily invalidate the use of the assay, but may help define the limitations of the assay.

**3.23****taxonomic authorities**

Literature references accepted by the relevant scientific community and providing the classification (e.g. family, genus, species) for a group of organisms.

**3.24****taxonomic identification**

The classification of an organism to family, genus, species, etc. These analyses are based on class characters diagnostic for the taxonomic level in question.

### **3.25 validation**

The process of performing and evaluating a set of experiments that establish the efficacy, reliability, and limitations of a method, procedure, or modification thereof; establishing recorded documentation that provides a high degree of assurance that a specific process will consistently produce an outcome meeting its predetermined specifications and quality attributes.

### **3.26 variation (interspecific)**

The diversity present in a character or set of character states that occurs between different species .

### **3.27 variation (intraspecific)**

The diversity present in a character or set of character states that occurs within the same species.

## **4 Requirements**

**4.1** If it is determined that a particular area listed below is not applicable to the primers/method being validated, an acceptable narrative of the basis of this determination shall be present in validation documentation.

**4.2** If validation reveals that the new marker, in any of the areas addressed below, is not suitable for the intended use, the new marker should not be used, unless an acceptable narrative of the reason for use is presented.

**4.3** The following areas should be addressed in a validation of new primers for amplification and sequencing.

**4.3.1** Characterization of loci—The following characteristics of a genetic region should be determined and documented, including:

- a) the location of the genetic region (nuclear or mitochondrial target);
- b) the technological basis for identifying the genetic marker (for example, capillary electrophoresis, DNA sequencing, or hybridization assays);
- c) a description of any known heteroplasmy, cytosine-rich regions or other anomalies.

**4.3.2** Species specificity—The ability to detect information from non-targeted species should be determined, including:

- a) species likely to be present in the laboratory (for example, human or species of pets owned by laboratory staff);
- b) species likely to be present in combination with the species of interest on evidentiary items (for example, predator DNA mixed with targeted prey DNA);
- c) species closely related to the species of interest, to demonstrate appropriate phylogenetic coverage using reference materials that follow documented taxonomic authorities.



NOTE The detection of genetic information from non-targeted species does not necessarily invalidate the use of the assay, but may help define the limits of the assay.

**4.3.3 Sensitivity studies**—Assess the amplification of a range of DNA quantities to determine the expected results with DNA concentrations at the extreme high and low ends of a concentration gradient applicable to the sample type being tested.

NOTE Examples of values to be determined include dynamic range, ideal target range, and limit of detection.

**4.3.4 Case-type samples**—The ability to obtain reliable results should be evaluated using samples that are representative of those typically encountered by the testing laboratory for the species of interest.

**4.3.5 Population studies**—The distribution of polymorphisms within a genetic region for the population in question should be catalogued:

- a) for taxonomic identification applications, an assessment of the degree of intraspecific versus interspecific variation for the targeted genetic region should be performed;
- b) for mitochondrial haplotyping applications, a sufficient number of individuals should be sequenced to allow a confidence interval to be calculated for any inclusion.

NOTE Population samples may be difficult to obtain for some threatened or endangered species. In these instances, the limitations of any statistical calculations should be clearly explained.

**4.3.6 PCR-based studies**—The following are required to demonstrate reproducibility, specificity and robustness.

- a) Primer sequences should be readily available. If this is not possible, a clear narrative of explanation should be present in validation documentation.
- b) The reaction conditions needed to provide the required degree of specificity and robustness should be evaluated. These include, but are not limited to, thermal cycling parameters, and the concentration of primers, magnesium chloride, DNA polymerase, and other critical reagents.
- c) Verify that the target region is amplified with minimal cross amplification of non-target areas and that the amplicon can be reliably sequenced.

## **Annex A** **(informative)**

### **Foundational Principles**

Differences in evolutionary histories for different taxonomic groups encountered in wildlife forensics often necessitate the use of multiple primer sets for multiple loci. Since this is a regular occurrence, laboratories need to be able to validate new primer sets in a straightforward and clearly defined way. Existing developmental and internal validation documents are all-encompassing and further guidance and details are required for laboratories who routinely need to validate new primer sets for sequencing.

Expansions of methodology are based on previously validated foundational principles (2, 7), methods, and reagents, so the validation process for an expansion often requires an abbreviated developmental validation process and an internal validation. Because these two validations are completed in the same laboratory, the overlap between developmental and internal validation is extensive. Therefore, these standards cover validation, generally, and do not delineate between developmental and internal validation.

## **Annex B**

### **(informative)**

## **Bibliography**

This is not meant to be an all-inclusive list as the group recognizes other publications on this subject exist. Additionally, any mention of a particular software tool or vendor as part of this bibliography is purely incidental, and inclusion does not imply endorsement by the ASB.

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