HOKLAS Supplementary Criteria No. 43

'Chinese Medicine' and 'Food' – Species Identification by DNA Sequencing for Authentication Purpose

0 Introduction

(a) This document serves to clarify and supplement the requirements of ISO/IEC 17025:2017 and HKAS PD001 for the accreditation of species identification by DNA sequencing. This document shall be read in conjunction with ISO/IEC 17025:2017, HKAS PD001 and the relevant HKAS and HOKLAS supplementary criteria documents.

(b) For the purpose of authentication, it is often necessary to identify the species from which food or Chinese materia medica (CMM) sample originates. Owing to the nature of this technique, the scope of accreditation for authentication purpose is restricted to food and CMM that are composed of the whole or a part of the body of a single organism. Food and CMM samples shall either be unprocessed or have only been subjected to processing that does not introduce contaminating DNA from other species. Laboratories using this method for authentication purpose shall be fully aware of these limitations. Laboratories shall obtain information such as the type and degree of processing of received samples from customers to determine the applicability of this technique to species authentication.

(c) Laboratories should also note that fulfilling the requirements in this document might not necessarily meet all the requirements of all test standards. Individual test standards may have specific requirements, which shall be met when conducting the tests.

1 Scope

(No additional explanation)

2 Normative references

(No additional explanation)

3 Terms and definitions

(No additional explanation)

4 General requirements

(No additional explanation)

5 Structural requirements

5.1 The laboratory shall have at least one staff member who possesses adequate knowledge of and experience in molecular analytical techniques, such as application of thermal cycler, Polymerase Chain Reaction (PCR) and DNA sequencing. He/she shall be responsible for the technical operation with respect to DNA sequencing in the laboratory.

6 Resource requirements

6.1 General

(No additional explanation)

6.2 Personnel

(a) Testing shall be performed by staff members who have undergone formal training in molecular analysis. A training programme shall include, in addition to the test procedures, training on sampling and handling of samples, prevention of cross-contamination, data handling and quality control techniques. Training materials should be documented and authorised. Full records of training and competence assessments shall be maintained.

(b) HOKLAS approved signatories

(i) HOKLAS approved signatories shall have as a minimum a bachelor degree in molecular biology, biology, biochemistry or other relevant subjects and possess relevant testing experience of not less than three years. Appropriate membership of professional bodies is also acceptable in lieu of academic qualifications. In addition, they shall have at least six-month experience in DNA sequencing.

(ii) For HOKLAS approved signatories signing test reports containing results on species identification of food samples, they shall, in addition to clause 6.2(b)(i), have adequate knowledge of taxonomy and food science and technology. Similarly, approved signatories signing test reports on CMM samples shall have adequate knowledge of taxonomy and Chinese medicine pharmaceutics in addition to clause 6.2(b)(i).

Note: Special consideration may be given to persons without the above qualifications but with extensive experience (i.e. at least 10 years) in DNA sequencing.

(iii) In all cases, candidates shall demonstrate to the assessors their technical competence in DNA sequence analysis, knowledge on taxonomy and where relevant knowledge of food science and technology or Chinese medicine pharmaceutics before signatory approval can be granted.

6.3 Facilities and environmental conditions

(a) The laboratory shall be clean and have adequate lighting at bench tops. Temperature and humidity shall be controlled and their acceptable ranges shall be defined and documented. There shall be effective separation of the test area from neighbouring laboratory areas to minimise contamination from nucleic acids and/or nuclease (both DNase and RNase). Contamination in major testing areas shall be regularly monitored (e.g. by analysing ‘open-tube’ blanks or bench surface swabs) and related records shall be maintained. Appropriate corrective actions shall be taken if contamination is detected. Procedures and precautions taken in avoiding cross-contamination shall be documented. Such procedures shall include washing of labware, generation of distilled, deionised or reagent water, decontamination of equipment between samples during PCR analysis, cleaning of work surfaces and other relevant activities.

(b) Where the areas for preparation of reagents and samples are located in the same room, distinct separation of these activities shall be maintained and appropriate procedures and control shall be adopted to prevent cross-contamination.

(c) Reagents, consumables and equipment shall be located at appropriate designated areas to serve their specific purposes. DNA extracts shall be

kept in designated refrigerated compartments after sample preparation. They shall not be kept in areas where activity such as gel electrophoresis or PCR work is conducted.

(d) At least 5 separate rooms or clearly designated areas with appropriate measures to prevent cross-contamination shall be provided for the following processes:

(i) sample receipt;
(ii) sample preparation and extraction;
(iii) reagents preparation and dispensing of master mix;
(iv) amplification, product detection; and
(v) Post-PCR product purification and DNA sequencing.

If separated designated rooms or areas dedicated for different operations are not possible, distinct separation of these activities shall be maintained and appropriate procedures and controls shall be used to prevent cross-contamination and their effectiveness shall be proved. The test environment shall also be monitored for possible cross-contamination.

(e) Separate enclosures shall be provided for the storage of the following materials:

(i) certified reference materials;
(ii) negative control materials;
(iii) positive control materials;
(iv) test samples;
(v) kits, master mix, DNA polymerase, primers, probes, reagents;
(vi) DNA extracts after sample preparation; and
(vii) PCR/sequencing products.

(f) A forward flow principle for sample handling shall be followed. The movement of nucleic acid samples, specimens or DNA extracts shall be unidirectional (i.e. from pre-amplification to post-amplification areas). Arrangement shall be made to ensure that any materials (e.g. printouts, stationery, etc.) in/from post-amplification areas are protected from cross-contamination.

(g) Staff shall wear lab coats and disposable gloves at work areas. Gloves and lab coats should be changed at appropriate frequency, e.g. between handling pre-amplification materials and post-amplification materials.

6.4 Equipment

(a) General equipment for molecular analysis includes balances, thermometers, pH meter, micropipettes, timer, vortex mixer, spectrophotometer, centrifuges and volumetric labware. Performance and precision of these items of equipment shall meet the specifications of the tests. The requirements relating to maintenance, verifications and calibrations of these items of equipment described in HOKLAS SC-02 shall apply.

(b) Automatic liquid handling system for DNA extraction and PCR set-up shall be verified regularly to ensure that their performance, including accuracy and precision, complies with the specifications of the tests.

(c) The performance of thermal cyclers, DNA sequencers and the corresponding built-in spectroscopic components shall be verified regularly to ensure that the tolerances required by the tests performed are complied with. Verifications or calibrations of these equipment shall be performed at least once a year, or at a frequency recommended by the manufacturer, whichever is more frequent. Critical parameters that have effects on the validity of test results such as temperature and time shall be calibrated. Temperature and optical signal at all reaction wells, where applicable, shall be verified. Procedures and records of performance checks, maintenance activities, verifications and calibration shall be available.

(d) Micropipettes shall be designed to prevent the possibility of aerosol production. Aerosol resistant pipette tips or positive displacement pipettes are strongly recommended. General instruments such as vortex mixers and micro-centrifuges should be placed at as great a distance from sample preparation work place as possible. Thermal cyclers and DNA sequencers shall be placed at designated areas.

(e) Chemicals and reagents involved from sample preparation down to testing shall be of molecular biology grade or equivalent. They shall also be certified or tested to be free from nucleic acids and nuclease (both DNase and RNase).

(f) Any special precautions in the preparation or use of the reagents, including relevant information on the stability of reagents to heat, air, light and other chemicals, etc., shall be documented. Reagents prepared in laboratory

shall be readily identifiable with strength, types of solvent used, dates of expiration and preparation. Preparation procedures and records shall be available. Persons responsible for preparation of reagents shall also be identifiable. Any special precautions, hazards or restrictions in using the reagents shall also be indicated. Laboratory personnel should take necessary precautionary measures when handling carcinogenic or toxic reagents.

(g) The sources and histories of consumables having an effect on the validity of test results, such as extraction kits and DNA polymerase, shall be documented. Records on information such as supplier, lot number, date of receipt, date put in use, date of verification and date of expiration of all critical reagents shall be maintained. The critical reagents used shall be traceable in the test records.

(h) All extraction kits, PCR kits, sequencing kits, enzymes, oligonucleotide primers, dye terminators and sequencing polymer shall be checked for their physical conditions and verified for their performance prior to release for use. Descriptions of the nature and sequence of primers and probes shall be provided. Verification procedures, criteria for acceptance, shelf life, and special storage conditions shall be documented. Records of verification and monitoring of the storage conditions shall be maintained.

(i) The software and/or computer programmes used for data analysis shall be verified and recorded.

6.5 Metrological traceability

(a) A suitable reference material is generally required for validation of a method. There are different kinds of reference materials for use in methods for detection of target DNA sequences. Reference materials can be substances in original form or in the form of genomic and plasmid DNA.

(b) Laboratories shall demonstrate the authenticity of the specimens used for the determination of the reference DNA sequences by using certified reference materials obtained from a reputable national institute or authority, such as the Natural History Museum (UK), Australian Museum, Muséum national d'Histoire naturelle (France), Naturalis Biodiversity Center (Netherlands) and National Museum of Natural History - Smithsonian Institution (USA), National Institutes for Food and Drug Control (China).

Reference materials not obtained directly from but authenticated by a recognised national institute or authority may also be accepted. Policies and procedures for purchasing, handling, storage, verification, preservation, maintenance and use of reference materials and stocks shall be documented. Detailed records shall be maintained.

(c) When certified reference materials or appropriately authenticated reference materials are not available, reference DNA sequences obtained from public genetic sequence databases, such as the Barcode of Life Data System (BOLD), GenBank or RefSeq of National Center for Biotechnology Information (NCBI), Reference DNA Sequence Library for Chinese Materia Medica (CMMRSL) from the Government Chinese Medicines Testing Institute (GCMTI) of HKSAR may be used for homology matching, on condition that:

(i) the reference DNA sequence shall be independently submitted by at least two different groups of researchers and the homology of the two submissions shall be 100%; or

(ii) if (i) cannot be met, the reference DNA sequence shall be submitted by a recognised laboratory which has published significant work on the target species/varieties and the method used for obtaining the sequence is based on Sanger sequencing.

Note: Methods based on Next-Generation Sequencing (NGS) may be used provided that the sequencing quality shall reach a base call accuracy of 99.9% or above, which is equivalent to Phred Quality Score 30 or above.

(d) To infer homology, DNA sequences/genetic patterns of the tested material shall match with those of the reference (either determined from reference materials or obtained from public genetic sequence databases) at 98% or higher. Such matching criteria shall be set based on the actual validation data obtained.

6.6 Externally provided products and services

(No additional explanation)

7 Process requirements

7.1 Review of requests, tenders and contracts

(No additional explanation)

7.2 Selection, verification and validation of methods

7.2.1 Selection and verification of methods

(a) Laboratories shall preferably use national, regional, and international standard methods, or standard methods published by reputable professional bodies. Laboratories may also use standard test methods outside their scope of application and laboratory-developed methods but they have to be validated. Laboratories shall demonstrate that each particular method is suitable for its intended purpose and the needs of the customers are met. When standard methods are used, laboratories shall verify their ability to achieve satisfactory performance against the documented performance characteristics of the method by using certified reference materials or participation in relevant proficiency testing (PT) programmes. Laboratories shall normally adhere to the limitations, concentrations range and sample matrix specified in the test standards.

(b) Physical characteristics of the test sample (including appearance, smell, texture etc.,) may help to provide additional information on the identification of its species. Such examination should be performed prior to DNA sequence analysis wherever possible and any useful information obtained should be recorded. If physical characteristics noted clearly do not match the species claimed, the laboratory shall seek clarification from the customer before proceeding with testing. The laboratory shall also check whether the conclusion drawn from DNA sequencing matches with those physical characteristics noted.

(c) There are some types of test samples for which DNA sequencing alone cannot provide confirmative authentication. These include, for example, test samples that are not a body part of a single organism, do not normally contain DNA material, have undergone processing that would introduce contaminating DNA, or of which DNA has degraded significantly. In this case, the laboratory shall employ other methodologies e.g. morphological identification or chemical analysis for authentication, which are not covered by this document, while results from DNA sequencing can only

serve as supportive evidence.

(d) Selection of target sequence

(i) For the purpose of authentication, laboratories shall select target sequence(s) that unequivocally detects and identifies a specific organism/species which could be mixed with similar organisms/species. Supporting information shall be documented.

(ii) Selection criteria shall include, but not limited to, the discriminatory power against different species/strains/varieties, repeatability and reproducibility, and possibility of 'null' alleles or allele competition. Sequences that share high homology in closely-related species should be avoided.

(iii) The length of target sequence shall be carefully selected to maximise specificity. In general, a sequence length of about 500 base pairs is adequate for species-level identification for animal-based samples, though this may vary among different species or sample conditions. For other samples (e.g. plant-based samples), a shorter sequence length may be acceptable, provided that proper justifications can be furnished. The laboratory shall ensure that the selected fragment covers sufficient sequence length for species-level identification, and supporting evidence shall be maintained. Where possible, a combination of two or more loci shall be used if this can significantly enhance the discriminatory power of the test.

(e) When performing sequencing, PCR products shall be sequenced bidirectionally, whenever possible.

7.2.2 Validation of methods

(a) Pre-validation preparation

(i) A general description of all components of the method shall be provided, including but not limited to the scope of the method and the unit of measurement. The purpose of the method shall also be indicated.

(ii) An overview of scientific principles and background of the method

shall be documented. Extensive literature review on proposed methods should be conducted to assess the suitability for the intended purpose.

(iii) Details of the proposed analytical method, including the sequence of oligonucleotide primers and probes, the length of target sequence, enzymes and reagents involved, nucleic acid extraction and detection methods shall be documented. Control samples and standards shall also be defined. Laboratories shall record clearly the rationale for the selection of each critical parameter.

(iv) If a prediction model or mathematical model was used, such model should be scientifically sound. The model shall be outlined and recorded, and instruction for correct application shall be provided. It shall be subjected to validation together with the analytical method.

(b) Method validation

(i) Laboratories shall demonstrate the selectivity of methods. Appropriate negative controls shall be determined. Methods shall be tested with DNA from non-target closely-related species and reference species to illustrate the degree of selectivity and to determine the false positive rate.

(ii) Inclusivity assessment of the method shall be conducted with positive samples of different origins (e.g. geographical) as far as possible. Genetic diversity of species should be maximised in selecting positive samples for the assessment.

(iii) Laboratories shall conduct tests at different concentrations of DNA to determine the false negative rate of methods. The limit of detection (LOD), defined as the concentration at which a positive sample yields a positive result at least 95% of the time, shall be determined. Observed inhibition of DNA amplification and sequencing shall also be analysed and identified.

(iv) Laboratories shall determine the applicability of methods in test samples that have been processed, e.g. sundried or frozen, if it is to be included in the intended scope of testing. Relevant limitations should be addressed.

(v) Laboratories shall also validate the extraction methods for their ability to extract DNA and remove inhibiting substances.

(vi) The minimum amount of PCR products required for successful sequencing reaction shall be determined during method validation.

(vii) Experimental design shall be fully documented. It should include, but not limited to, amount of starting material, dilutions and master mix composition. Laboratories shall also define all conditions, including the apparatus used, under which critical steps of the methods are performed. Since some methods are instrument or chemistry specific, validated methods shall be strictly followed. Any substitution of alternative processes shall be re-validated to show that it will not affect the performance of the original method.

(viii) Laboratories shall demonstrate that selected methods are repeatable (within run) and reproducible (between runs using different extracts).

(ix) To facilitate the routine application of methods, it could be helpful for laboratories to provide information on operational characteristics and practicability. A description of practical skills shall be provided if they are necessary for properly applying the methods.

(x) Proprietary test systems (kits) may not require further validation if validation data based on collaborative testing are available. Otherwise, the laboratory shall be responsible for validating such systems. The laboratory shall demonstrate their capability to achieve the limit of detection quoted by the manufacturer and that false positives and negatives shall not occur.

7.3 Sampling

(a) Sampling from a sample lot or site is not covered in this document. Customers taking their own samples shall be made aware of proper storage, sampling and transportation procedures.

(b) When the sample submitted by customers contains individual items, the items shall not be mixed for testing. Each item shall be tested separately and reported as an individual sample. In cases where not all

items in the sample are tested, the number of items selected for testing and the total number of items received by the laboratory shall be recorded and reported. If the items show variation in physical appearance or it is suspected that the items are not homogeneous, it is not appropriate to report a result that relates to the entire sample as received. These items shall be tested separately instead.

(c) Laboratories shall document the minimum sample size particularly for raw materials and sub-sampling procedures for taking test portions from laboratory samples. Laboratories shall have statistically valid sub-sampling plan to ensure that the test portion is representative of the laboratory sample, and the composition of the sample shall not be altered in a way that would affect the concentration of the target DNA. Preparation of laboratory samples and test portions, if not specified in test standards, should be based on national or international standards specific to the sample tested. Customers should be informed if the sample size received is too small for meaningful analysis.

7.4 Handling of test items

(a) Laboratories shall examine and record the conditions and appearance of samples upon receipt. Items to be checked include nature and characteristics of sample, volume/amount of sample, conditions of sample container, characteristics of the sampling operation (sampling date and condition), etc. Any deviation from the specified conditions shall be handled in accordance with Cl. 7.4.3 of ISO/IEC 17025:2017.

(b) Samples pending testing shall be stored under suitable conditions to minimise any degradation. Storage conditions for samples and sample extracts shall be documented and shall fulfil the requirements of test standards.

(c) Frequently, it is necessary to split or transfer samples for testing of different properties. It is essential that procedures are available for preventing contamination. Documented procedures and records on delivery of samples including special transportation such as refrigeration and exclusion of light, disposal and decontamination processes and unbroken chain of identification of the sub-samples/samples shall be available.

7.5 Technical records

(No additional explanation)

7.6 Evaluation of measurement uncertainty

(No additional explanation)

7.7 Ensuring the validity of results

(a) Laboratories shall establish and implement quality control plans to ensure and demonstrate that the measurement process is in-control and test results generated are accurate and valid. The plans shall include types of quality control checks, their frequencies and acceptance criteria, and actions to be taken when results are out of control limits.

(b) Materials from PT schemes, previously tested and verified positive samples, genomic DNA or plasmid DNA may be used as in-house quality control materials.

(c) Quality control plans that are stipulated in test standards shall be followed strictly. If such plans are not given, the following controls shall be run, where appropriate, at a minimum of once for every test run:-

(i) Extraction negative (or blank) control
The extraction buffer employed for all steps of extraction procedure, without addition of the test material, shall be prepared e.g. autoclaved sterile water.

(ii) PCR/Sequencing negative control
Master mix without DNA template shall be included as negative control.

(iii) Extraction positive control
Control sample known to contain the target sequence shall be included in DNA extraction. Quality of the extracted nucleic acids from all samples shall also be assessed by well-established methods (e.g. gel based assessment and amplification of a taxon-specific reference sequence).

(iv) PCR/Sequencing positive control
Reference DNA or DNA extracted from a CRM or a known positive sample representative of a gene sequence under study. It shall be incorporated to demonstrate the unique performance of the assay.

(v) Repeat testing (if applicable)
If no PCR product is obtained from a test sample, such PCR shall be repeated to avoid false negative result.

(d) As PCR technique is prone to contamination, laboratories shall be aware of any possible contamination of the sample. For example, if the amount of the target sequence in the sample after amplification is found to be abnormally low and it is not due to poor quality of DNA material or PCR inhibitions, it may indicate that the sample contains DNA from non-targeted species arising from contamination.

(e) The overall quality of the chromatogram data shall be assessed.

(i) If the chromatogram demonstrates the following: the limit of resolution is reached, a lack of extension products (i.e. no peak), multiple sequences/peaks, a rapid decline, or 'the spread', which indicates a condition not suitable for analysis, the sample shall be re-extracted, re-amplified, and/or re-sequenced.

(ii) If the chromatogram has unincorporated nucleotide peaks, dye blob, pull up, mobility errors, or background noise, the sequence shall be edited manually and records of editing shall be maintained.

(f) Laboratories shall participate in appropriate PT activities where applicable.

(i) Frequency of participation shall commensurate with the outcome of the laboratory's risk assessment and shall be at least once per year for each area of technical competence, as defined by a minimum of one measurement technique, parameter and matrix which are related (please refer to Appendix C of ILAC-P9:01/2024 for more details).

(ii) Laboratories shall document procedures for rectifying unsatisfactory performance in PT activities. If unsatisfactory results are obtained, the laboratory shall promptly investigate the root cause(s), take

action(s) to rectify the problem(s) and demonstrate that it can achieve satisfactory performance for the test/method in question. All findings and actions taken in connection with unsatisfactory performance shall be recorded.

7.8 Reporting of results

(a) Test reports shall be clear and not be potentially misleading.

(b) For authentication of a test sample that is composed of a whole organism or a body part of an organism, a test report may state that such sample originates from the species identified. For example, the result may be given as:

'The sample is found to be *Species X* in origin based on the results of DNA sequencing'.

In case of non-matching result, an example of the result may be:

'The species origin of the sample cannot be ascertained by the results of DNA sequencing'.

(c) For a test sample other than those described in clause 7.8(b), or for a test sample for which DNA sequencing alone cannot provide confirmative authentication (clause 7.2.1(c)), laboratories shall not report the species from which the sample originates even if it contains DNA that match the sequence of a particular species. The laboratory shall clearly explain in the report that DNA sequence analysis alone is not sufficient for authentication purpose in this circumstance. An appropriate conclusion may be:

'The sample was found to contain DNA from *Species X* based on the results of DNA sequencing'.

(d) If a conclusion cannot be drawn due to insufficient amount or poor quality of extracted DNA or other technical reasons, it shall be stated clearly in the report.

(e) Due to the limitation of this method, test result can apply only to the sample tested and this fact shall be stated in the report. If the sample received contains many individual items and only some are selected for

testing, a description on the selection procedure shall be given, e.g. 5 individual items were selected randomly and tested separately. If there is variation in physical appearance of individual items in the sample, or inhomogeneity amongst items is suspected, results on the individual items shall be reported instead. Information provided by the customer shall also be given in the report in as much detail as possible.

(f) The sample preparation procedure shall be given if it is required for the proper interpretation of test results.

7.9 Complaints

(No additional explanation)

7.10 Nonconforming work

(No additional explanation)

7.11 Control of data and information management

(No additional explanation)

8 Management system requirements

(No additional explanation)

Annex
(Informative)

Bibliography

1 ILAC-P9:01/2024 *ILAC Policy for Proficiency Testing and/or Interlaboratory Comparisons other than Proficiency Testing*

2 U.S. Food and Drug Administration Foods Program, Edition 1.2, Dec 2023 *Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of Food, Feed, Cosmetics and Veterinary Products*

3 中國藥典 9107 中藥材 DNA 條形碼分子鑒定法指導原則

4 ISO 21286 *Soil quality – Identification of ecotoxicological test species by DNA barcoding*

5 BS EN ISO 17174: 2024 *Molecular biomarker analysis – DNA barcoding of fish and fish products using defined mitochondrial cytochrome b and cytochrome c oxidase I gene segments*

6 BS EN 17477:2021 *Algae and algae products — Identification of the biomass of microalgae, macroalgae, cyanobacteria and Labyrinthulomycetes — Detection and identification with morphological and/or molecular methods*

7 BS EN 17881: 2024 *Food authenticity – DNA barcoding of bivalves and products derived from bivalves using a defined mitochondrial 16S rRNA gene segment*

8 BS EN 17882:2024 *Food authenticity – DNA barcoding of meat derived from mammals and birds using defined mitochondrial cytochrome b and cytochrome c oxidase I gene segments*

9 Shaw, P.C., Cao, H and Lo, Y.T. (e.d.) (2023) *Authentication of Chinese Medicinal Materials by DNA Technology*, 2nd ed., Word Scientific Publishing Co, Ltd. Singapore

Remark: For dated references in the whole Annex, only the edition cited applies. For undated references cited, the latest edition (including any amendments) applies.