

Laboratory Information Bulletin

A Protocol for Validation of DNA-Barcoding for the Species Identification of Fish for FDA Regulatory Compliance

Haile F. Yancy¹, Frederick S. Fry², Spring C. Randolph², Jonathan Deeds², Natalia V. Ivanova³, Christopher M. Grainger³, Robert Hanner³, Lee A. Weigt⁴, Amy Driskell⁴, Jeffrey Hunt⁴, Andrea Ormos⁴ and Paul D.N. Hebert³

¹U. S. Food and Drug Administration, Center for Veterinary Medicine, Office of Research, 8401 Muirkirk Road, Laurel, Maryland 20708, USA

²U. S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD 20740, USA

³Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, University of Guelph, 579 Gordon Street, Guelph, ON, Canada, N1G 2W1

⁴Laboratories of Analytical Biology, National Museum of Natural History, Smithsonian Institution, MRC 534, Washington DC 20013-7012

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Abstract

A detailed protocol intended for validation is provided for DNA sequencing of the cytochrome c oxidase subunit I gene (COI) of seafood tissue samples for the purpose of obtaining unique, species-specific “barcodes” for species identification. These procedures include: Tissue Sampling, Tissue Lysis and DNA Extraction, Polymerase Chain Reaction - COI Amplification, PCR Product Check, Cycle Sequencing Reaction, and Sequencing Reaction Cleanup.

Introduction

Seafood is gaining increasing attention due to the potential health-related risks associated with misbranding species. It is vital that aquatic animals harvested, processed, distributed and sold in the U.S. as food are safe, wholesome, and properly labeled. Some examples of specific species related hazards are listed in the U.S. FDA Seafood Hazard Guide (Food and Drug Administration Fish and Fisheries Products Hazards and Controls Guidance (2001) [<http://www.cfsan.fda.gov/~comm/haccp4c.html>]).

Under the Federal Food, Drug, and Cosmetic Act (FD&C), the Fair Packaging and Labeling Act (FPLA) and the Public Health Service Act (PHS) the Food and Drug Administration facilitates programs that include inspection, sampling, analysis, research, and education on seafood issues. These programs assist the agency with its effort to oversee food safety and economic deception. The detection of species substitution is critical because it will assist in identifying and controlling specific species related hazards and reduce economic fraud.

One challenge faced by both consumers and regulators is the detection of "seafood substitution" in the marketplace - where low value species or a species with a potential food safety hazard are mislabeled and substituted in whole or in part for a more expensive, or for a species with no potential food safety hazard.

It is not always possible to tell just by simple inspection of an aquatic product that misbranding has occurred. Processing often removes or damages diagnostic characteristics crucial for the identification of species by conventional taxonomic means. Therefore, traditional morphological methods are often insufficient to provide for species resolution identification. An example of this problem is highlighted in a study by Marko et al (Marko et al 2004) which showed that 77% of Red Snapper fillets sold in the eastern USA were mislabeled.

Several years ago, a web-based resource known as the Regulatory Fish Encyclopedia (RFE) was developed for the identification of commercially important species of fishes, (Tenge et al. 1997). Organized in a series of species “pages”, the RFE contains high resolution images of whole fish and their marketed product forms (e.g. fillets, steaks), as well as other taxonomic, geographic, and relevant tools for species identification. An example of an identification method listed on the website is protein identification by isoelectric focusing (Regulatory Fish Encyclopedia (RFE) Isoelectric Focusing (IEF) Peak Report (PKRPT) 2000

[<http://www.cfsan.fda.gov/~frf/rfe5prsm.html>]). Isoelectric focusing is the currently accepted tool employed in the identification of fish fillets for regulatory compliance, but such analysis requires subjective interpretations of gel results and the inclusion of several standards in each run. Further, the technique is not effective in the case of processed or cooked samples. The RFE was designed so that it could be expanded to include additional data and to accommodate the use of newer analytical tools as they became available. Recently, DNA barcodes were generated for 172 individual authenticated fish representing 72 species from 27 families contained in the RFE (Yancy et al. 2007).

The Barcode of Life initiative represents an ambitious effort to develop an identification system for eukaryotic life based upon the analysis of sequence diversity in short, standardized gene regions. Work is furthest advanced for members of the animal kingdom. In this case, a target gene region has been selected (cytochrome *c* oxidase 1) and pilot studies have validated its effectiveness in species discovery and identification. Even relatively short nucleotide sequences (100-200 bp) from the barcode region provide accurate identifications, allowing barcodes to identify specimens whose DNA is degraded (Hajibabaei et al. 2006). The benefits of this work include facilitating species identification, flagging previously unrecognized species, and enabling identifications where traditional methods are not applicable, such as for immature stages or body fragments.

The Fish Barcode of Life project (FISH-BOL [<http://www.fishbol.org>]) is a collaborative international research effort which seeks to establish a reference library of DNA barcodes for all fish species derived from voucher specimens with authoritative taxonomic identifications (Hanner et al. 2005). Fishes comprise nearly half of all vertebrate species; the group includes approximately 15,700 marine and 13,700 freshwater species (FishBase [<http://www.fishbase.org>]). The FISH-BOL database now includes records for 3960 species of fish represented by 19230 specimens. Once completed, the database will enable a fast, accurate, and cost-effective system for molecular identification of the world's ichthyofauna.

The Canadian Centre for DNA Barcoding (CCDB) has developed a well-regimented, largely automated protocol for DNA barcode analysis. The standard fish barcoding analytical chain at CCDB includes DNA extraction using a Glass-Fiber protocol (Ivanova et al. 2006), PCR amplification using pre-made PCR plates (Hajibabaei et al. 2005, Ivanova & Grainger 2007) with primer cocktails that allow the amplification of all fish species (Ivanova et al. 2007) and cycle-sequencing with M13 primers using pre-made sequencing plates (Hajibabaei et al. 2005, Ivanova & Grainger 2007). Sequencing products are analyzed on a 3730xl DNA analyzer (Applied BioSystems) and resulting trace files are edited and aligned using Sequencher 4.5 (GeneCode Corporation). Sequences are then analyzed using the BOLD identification engine (Ratnasingham & Hebert 2007).

The US FDA Center for Veterinary Medicine in collaboration with the Center for Food Safety and Applied Nutrition, the Biodiversity Institute of Ontario at the University of Guelph, Ontario, Canada, and the Laboratories of Analytical Biology at the Smithsonian National Museum of Natural History, Suitland, MD, USA provide details on protocols, reagents and equipment required to carry out a validation study for barcode generation for fish identification., The process is separated into six major steps in the analytical pathway:

1. Tissue Sampling
2. Tissue Lysis and DNA Extraction
3. Polymerase Chain Reaction- COI Amplification
4. PCR Product Check
5. Cycle Sequencing Reaction
6. Sequencing Reaction Cleanup

Important Note: The end product of this procedure will be unique, species-specific “barcode” sequences of the mitochondrial COI gene that can be used for species identification of fish. Interpretation of these results, i.e. barcode alignment and species identification using a database of barcodes based on authenticated standards, will be described in a separate document.

1. TISSUE SAMPLING

1A. Reagents

- a. Reagent Alcohol, Histological (EtOH 96%); Fisher Scientific Catalog No. A962-4

1B. Consumables

- a. TrakMates 2D Barcoded Screwtop tubes in latch racks; Matrix Technologies Catalog No. 3741

1C. Protocol for Tissue Sampling

Several tissues are suitable for DNA extraction from fishes. These include:

- a. Musculature: remove one or more cubes (5-7 mm) of lateral muscle (skin removed) from the right side of the specimen (if whole).
- b. Fin clips: remove fin rays and membrane from the right pectoral or pelvic fin. Use these tissues only if the fish needs to be kept alive.
- c. Eye: remove the right eye from extremely small specimens such as larvae.

Note: The use of tissues from the right side of the animal is due to the fact that most taxonomists prefer the left side of the animal intact for visual identification. There is no biological reason for using tissues from the right side of the animal exclusively.

Tissues samples should be removed with a scalpel and forceps that are flame sterilized after each fish is processed. Tissues should be frozen at -20°C or preserved in fresh 95% ethanol and stored in a cool place, preferably in a freezer until DNA is extracted. Tissue samples can be conveniently stored in batches of 96 in Matrix boxes.

2. TISSUE LYSIS AND DNA EXTRACTION

Numerous published methods and commercial kits are available for tissue lysis and DNA extraction. Any method that yields quality DNA suitable for PCR and sequencing is potentially acceptable. In the appendix we describe four methods for DNA extraction: (1) DNeasy Blood & Tissue Kit Qiagen Catalog No. 69504 (50) or 69506 (250) (U. Guelph). (2) Automated DNA extraction on Beckman/Coulter Biomek FX (U. Guelph). (3) BioSprint 96 DNA Blood Kit (Qiagen) (Smithsonian Inst.). (4) AutoGenPrep 965 (Smithsonian Inst.)

These methods have been evaluated by the participating laboratories and found to be acceptable for the production of DNA that is suitable for PCR and sequencing. Additional methods will need to be evaluated separately before they would be acceptable for inclusion in this LIB.

3. POLYMERASE CHAIN REACTION – COI AMPLIFICATION

3A. Consumables & Equipment

- a. Molecular grade water (dd H₂O); Invitrogen Catalog No. 10977023
- b. D – (+) – Trehalose dihydrate; Sigma Catalog No. T9531-100g
- c. 10X PCR Buffer, Minus Mg; Invitrogen Catalog No. 10966-034
- d. 50 mM Magnesium Chloride; Invitrogen Catalog No. 10966-034
- e. Deoxynucleotide Solution Mix; New England Biolabs Catalog No. N0447L
- f. Oligonucleotide Primers; University of Guelph, Laboratory Services.
- g. Platinum Taq DNA Polymerase; Invitrogen Catalog No. 10966-034
- h. Eppendorf research series 2100 pipetter 2-20 µl; Fisher Scientific Catalog No. 05-402-87
- i. Eppendorf research series 2100 pipetter 20-200 µl; Fisher Scientific Catalog No. 05-402-89
- j. Eppendorf research 8-Channel pipetter 05-10 µl; Fisher Scientific Catalog No. 13-688-507
- k. Brand Transferpette 8-Channel pipetter 5-50 µl; Fisher Scientific Catalog No. 03-840-10
- l. Eppendorf research pro pipette 100-5000 µl; Fisher Scientific Catalog No. 21-378-84
- m. Eppendorf research 12-Channel pipetter 0.5-10 µl; Fisher Scientific Catalog No. 13-688-503
- n. Eppendorf research 12-Channel pipetter 10-100 µl; Fisher Scientific Catalog No. 13-688-504
- o. Eppendorf Mastercycler® ep gradient S Thermocycler; Eppendorf Catalog No. 950010045
- p. Eppendorf heat sealer; Eppendorf Catalog No. 951023078
- q. Aluminum Sealing Film; Axygene Scientific. VWR Catalog No. CA47734-816
- r. Clear Sealing Film; Axygene Scientific. VWR Catalog No. CA10011-116
- s. Heat-Sealing Film; Axygene Scientific. VWR Catalog No. CA89005-634
- t. 1-10 µl non-filter tips; Axygene Scientific. VWR Catalog No. CA10011-872
- u. Eppendorf twin-tec PCR plate; Fisher Scientific Catalog No. E951020460
- v. IEC CL40 swinging bucket centrifuge; Thermo Electron Corporation Catalog No. 11210923

- w. 1-10 μ l filter tips; Fisher Scientific Catalog No. CS004807
- x. 1-30 μ l filter tips; Fisher Scientific Catalog No. CS004821

3B. Polymerase Chain Reaction Recipe

In a 1.5 ml tube, mix M13-tailed primer cocktails:

C_FishF1t1 VF2_t1 : FishF2_t1 (1:1, 10 μ M each primer)

FishF2_t1 TGATAAACGACGGCCAGTCTGACTAATCATAAAGATATCGGCAC
VF2_t1 TGATAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC

C_FishR1t1 FishR2_t1 : FR1d_t1 (1:1, 10 μ M each primer)

FishR2_t1 CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA
FR1d_t1 CAGGAAACAGCTATGACACCTCAGGGTGTCGAARAAYCARAA

PCR REAGENTS

	Each well (μ l)	96-well plate (μ l)
1. 10% trehalose	6.25	625.0
2. dd H ₂ O	2.00	200.0
3. 10X buffer	1.25	125.0
4. 50 mM MgCl ₂	0.625	62.5
5. 10 μ M primer A	0.125	12.5
6. 10 μ M primer B	0.125	12.5
7. 10 mM dNTPs	0.062	6.2
8. Platinum Taq (5 U/ μ l)	0.060	6.0
Total	10.500	1050.0

3C. Protocols for Polymerase Chain Reaction

- a. Add all 8 reagents to a single 2 ml Eppendorf tube in the volumes listed under the “96 well plate”.
- b. This forms the “cocktail” of reagents for the PCR reaction. Vortex the cocktail vigorously or mix well by pipetting (Note: vortexing will cause liquid to be trapped on the cap of the tube. Before proceeding to the next step make sure that this liquid is returned to the rest of the cocktail by a 15 second spin in a mini centrifuge).
- c. Aliquot 130 μ l of cocktail into the last row of a 96-well plate using the single-channel 200 μ l pipette. Use the same tip for each transfer in this step.
- d. Transfer 10.5 μ l of cocktail from the last row to the wells in each row of the 96-well plate using the 8-channel 100 μ l pipette. Use the same tips for each transfer in this step.
- e. Aliquot 2 μ l of DNA template to each well of the 96-well plate. Use the 10 μ l 8-channel pipette to do this row by row. **Note:** change the tips after each row.
- f. Place aluminum foil or heat-seal cover over the top of the 96-well plate and centrifuge in an IEC CL 40 by holding the pulse button for approximately 10-15 seconds. Make sure that the centrifuge is properly balanced.
- g. Place the plate in a thermocycler block with a plastic mat on top of the plate and close the lid of the thermocycler.

- h. Start the program Fish52.
- i. Thermal conditions of Fish52: 94°C for 2 min, 35 cycles of 94°C for 30 sec, 52°C for 40 sec, and 72°C for 1 min, with a final extension at 72°C for 10 min.

Note: PCR plates can be made in batches, dispensed on a Beckman FX, and then stored frozen at -20°C until use. If using this approach, take a plate from a freezer, defrost it, spin for 1 min at 1000 g in the IEC CL 40 centrifuge and proceed to step 'e'.

4. PCR PRODUCT CHECK

4A. Consumables & Equipment

- a. AlphaImager® HP Gel documentation system; Alpha Innotech Catalog No. 92-13823-00
- b. 2% E-Gel® 96 Pre-cast agarose gels; Invitrogen Catalog No. G7008-02.
- c. E-Base® Integrated power supply; Invitrogen Catalog No. EB-M03
- d. Eppendorf research 12-Channel pipetter 0.5-10µl; Fisher Scientific Catalog No. 13-688-503

4B. Protocols for PCR Product Check

- a. Plug the Mother E-Base into an electrical outlet. Press and release the 'pwg/prg' (power/program) button on the base to select program EG. Select a run time of 6 min by pressing the 'time' button.
- b. Remove gel from the package and remove plastic comb from the gel. Slide gel into the two electrode connections on the Mother E-Base.
- c. Load 16 µl of dd H₂O into each well with 12-channel pipette.
- d. Load 4 µl of each PCR product into a different well with the 12-channel pipette.
- e. To begin electrophoresis, press the 'pwd/prg' button. The red light should change to green.
- f. At the end of run (signaled with a flashing red light and rapid beeping), press and release the 'pwr/prg' button.
- g. Remove the gel cassette from the base and capture a digital image of the gel with the Alpha Imager documentation system.
- h. Arrange lanes and manipulate image as necessary using the Invitrogen E-Editor software (available at <http://www.invitrogen.com/egels>).
- i. Incorporate the E-gel image into 'electronic lab book' spreadsheet.
- j. If the gel shows a success rate > 75%, the plate should be sequenced completely. Success rates below 75% require hitpicking of amplified products and a check for quality of extracted DNA for the failed samples utilizing 16S universal primers (Palumbi 1996) (PCR protocol identical to PCR I described above).

Universal 16S primers:

16Sar-5' CGCCTGTTTATCAAAAACAT
16Sbr-3' CCGGTCTGAACTCAGATCACGT

5. CYCLE SEQUENCING REACTION

5A. Consumables & Equipment

- a. Molecular grade water (dd H₂O); Invitrogen Catalog No. 10977023
- b. D – (+) – Trehalose dihydrate; Sigma Catalog No. T9531-100g
- c. 5X Sequencing Buffer (400 nm Tris-HCl pH 9.0 + 10 mM MgCl₂)
- d. Oligonucleotide Primer; University of Guelph, Laboratory Services.
- e. BigDye® Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems Catalog No. 4337457
- f. 1-10 µl filter tips; Fisher Scientific Catalog No. CS004807
- g. 1-30 µl filter tips; Fisher Scientific Catalog No. CS004821
- h. Eppendorf research series 2100 pipetter 2-20 µl; Fisher Scientific Catalog No. 05-402-87
- i. Eppendorf research series 2100 pipetter 20-200 µl; Fisher Scientific Catalog No. 05-402-89
- j. Eppendorf research 8-Channel pipetter 05-10 µl; Fisher Scientific Catalog No. 13-688-507
- k. Brand Transferpette 8-Channel pipetter 5-50 µl; Fisher Scientific Catalog No. 03-840-10
- l. Eppendorf research pro pipette 100-5000 µl; Fisher Scientific Catalog No. 21-378-84
- m. Eppendorf research 12-Channel pipetter 0.5-10 µl; Fisher Scientific Catalog No. 13-688-503
- n. Eppendorf research 12-Channel pipetter 10-100 µl; Fisher Scientific Catalog No. 13-688-504
- o. Eppendorf twin-tec PCR plate; Fisher Scientific Catalog No. E951020460
- p. AirClean® Systems Ductless PCR Workstation; Fisher Scientific Catalog No. 36 099 3859
- q. Aluminum Sealing Film; Axygene Scientific VWR Catalog No. CA47734-816
- r. Clear Sealing Film; Axygene Scientific VWR Catalog No. CA10011-116
- s. Heat-Sealing Film; Axygene Scientific VWR Catalog No. CA89005-634
- t. Eppendorf Mastercycler® ep gradient S Thermocycler; Eppendorf Catalog No. 950010045
- u. IEC CL40 swinging bucket centrifuge; Thermo Electron Corporation Catalog No. 11210923

5B. Cycle Sequencing Reaction Recipe

Reagents	Each well (µl)	96-well Plate (µl)
1. Primer M13F (or M13R)	1	98
2. 5X SEQ Buffer	1.875	183.75
3. BigDye	0.25	24.5
4. dd H ₂ O	0.875	85.75
5. 10% Trehalose	5	490
Subtotal	9	882
DNA Template	1-1.5	n/a
Total	10-10.5	n/a

Note: 5X Sequencing buffer is: 400 mM Tris-HCl pH 9.0, 10 mM MgCl₂ or 5X ABI sequencing buffer.

M13 primers (Messing 1983):

M13F (-21) TGTAACGACGGCCAGT
M13R (-27) CAGGAAACAGCTATGAC

5C. Protocols for Cycle Sequencing

- a. Add reagents 1-5 to a single 2 ml Eppendorf tube in the volumes listed under the “96 well plate”. This forms the “cocktail” of reagents for the PCR reaction
Note: BigDye is light sensitive and should remain in the freezer except when in use. (Never leave it out for longer than a few minutes). Repeat this step using the other primer. The end result is 2 cocktails, one containing the forward primer and the other containing the reverse primer.
- b. Vortex the cocktail vigorously. **Note:** vortexing will cause liquid to be trapped on the cap of the tube. Before proceeding to the next step make sure that the liquid has been returned to the rest of the cocktail by quick spin in a mini-centrifuge.
- c. Aliquot 115 µl of cocktail into the last row of a 96-well plate using the single-channel 200 µl pipette. Use the same tip only for those transfers involving a single cocktail (i.e. before transferring the second cocktail you must change the tip).
- d. Transfer 9.0 µl of cocktail from the last row into each row of the 96-well plate using the 8-channel 10 µl pipette for both cocktails. Use the same tip only for those transfers involving a single cocktail (i.e. before transferring the second cocktail you must change the tip).
- e. Aliquot 1-1.5 µl of PCR product into each well of the 96-well plate. Use the 10 µl 8-channel pipette to do this row by row. **Note:** change the tips after each row.
- f. Place aluminum foil or heat-seal cover over the top of the 96-well plate and centrifuge in the IEC CL40 at 1000 g for 1 min. Make sure that the centrifuge is properly balanced.
- g. Place the plate in a thermocycler block with a plastic mat on top of the plate and close the lid of the thermocycler.
- h. Start the program Seq3.1.
- i. After the program has finished, stop the thermocycler and store the plates at 4°C in a dark box to avoid degradation of the light-sensitive sequencing products.

6. SEQUENCING REACTION CLEANUP

6A. Consumables & Equipment:

- a. Sephadex® G50. Sigma Catalog No. 658080-500g
- b. Molecular grade water (dd H₂O). Invitrogen Catalog No. 10977023
- c. Multi-well filter plate: Acroprep 96 Filter plate; 0.45 µM GHP. PALL Corporation Catalog No. 5030.
- d. Pop-7™ Polymer for 3730xl DNA Analyzers. Applied Biosystems Catalog No. 4335611

- e. 3730xl DNA Analyzer Capillary Array, 50 cm. Applied Biosystems Catalog No. 4331246
- f. Running Buffer, 10X for 3730xl DNA Analyzers. Applied Biosystems Catalog No. 4335613
- g. MicroAmp 96-well reaction plate. Applied Biosystems Catalog No. N8010560
- h. Span-8 P250 non sterile tips. Beckman Coulter Catalog No. CABK379501
- i. Span-8 P20 sterile barrier tips. Beckman Coulter Catalog No. CABK379506
- j. Biomek® FXp Laboratory Automated Workstation. Beckman Coulter Catalog No. A31844
- k. Hydroclave MC8 Steam Sterilizer. Barnstead International Catalog No. 265608
- l. AirClean® Systems Ductless PCR Workstation. Fisher Scientific Catalog No. 36 099 3859
- m. IEC CL40 swinging bucket centrifuge. Thermo Electron Corporation Catalog No. 11210923
- n. 3730xl DNA Analyzer. Applied Biosystems Catalog No. 3730XL
- o. Matrix Impact2 pipettor, 15 µl-1250 µl (8-channel Matrix multichannel pipette); Matrix Technologies Catalog No. 2004
- p. Matrix Technologies 1250 µl Pipet Tip (Non-filter tip); Matrix Technologies Catalog No. 8046

Note: Sequencing plates can be made in batches, dispensed with a Biomek FX, and stored frozen at -20°C. If this approach is adopted, remove a plate from the freezer, defrost it, and spin down for 1 min at 1000 g before proceeding to step 'e'.

6B. Protocols for Sequencing Cleanup

- a. Measure dry Sephadex with the black column loader into a 350 µl PALL filter plate.
- b. Hydrate the wells with 300 µl of molecular grade water on the Biomek FX or using a Matrix multichannel pipette.
- c. Let the Sephadex hydrate overnight at -4C or for 3-4 hours at room temperature before use.
- d. Join the Sephadex plate with the collection plate and secure them with at least 2 rubber bands.
- e. Make sure the two sets weigh the same (adjust weight by using different rubber bands).
- f. Centrifuge at 750 rcf in the IEC CL40 for 3 min to drain the water from the wells.
- g. Add the entire volume of the sequencing reaction to the center of the Sephadex columns using either the Biomek FX or a 8-channel pipettor.
- h. Add 25 µl of 0.1mM EDTA to each well of the autoclaved 96 well plate.
- i. To elute the clean sequencing reaction, attach the 96 well plate with the aliquoted EDTA to the bottom of the Sephadex plate and secure them with rubber bands.
- j. Make sure the sets weigh the same (adjust weight by using different rubber bands).
- k. Centrifuge at 750 rcf in the IEC CL40 for 3 min. Remove the Sephadex plate.
- l. Cover the top of collection plate with a septum.
- m. Place the 96 well plate into the black plate bases and attach the white plate retainer.
- n. Stack the assembled plate in the ABI 3730xl capillary sequencer and import the plate record.

7. REFERENCES

- BOLD Identification System (IDS) Identification Engine (2007). <http://www.boldsystems.org/views/idrequest.php> (page accessed on October 19, 2007).
- Food and Drug Administration Fish and Fisheries Products Hazards and Controls Guidance, Third Edition (2001), <http://www.cfsan.fda.gov/~comm/haccp4c.html> (page accessed October 11, 2007).
- FishBase (09/2007), <http://www.fishbase.org> (page accessed on November 27, 2007).
- FISH-BOL - Fish Barcode of Life project, <http://www.fishbol.org> (page accessed on November 27, 2007).
- Hajibabaei M, deWaard JR, Ivanova NV et al. (2005). Critical factors for assembling a high volume of DNA barcodes. *Philosophical Transactions of the Royal Society: Biological Sciences* **360**:1959-1967.
- Hanner RH, Schindel DE, Ward RD, Hebert PDN (2005). FISH-BOL Workshop Report, August 26, 2005. For the workshop held at the University of Guelph, Ontario, Canada June 5-8, 2005, <http://www.fishbol.org/news.php> (page accessed August 1, 2006).
- HsienYHP, Woodward BB, Blanko AW (1995) Species substitution of retail snapper fillets. *Journal of Food Quality* **18**: 131-140.
- Ivanova N, Grainger C (2006) Pre-made frozen PCR and sequencing plates. CCDB Advances, Methods Release No. 4. Biodiversity institute of Ontario.
- Ivanova NV, deWaard JR, Hajibabaei M, Hebert PDN (2005). Protocols for high volume DNA barcoding. Draft submission to: DNA working group Consortium for the Barcode of Life. Published online at http://barcoding.si.edu/PDF/Protocols_for_High_Volume_DNA_Barcode_Analysis.pdf (page accessed October 19, 2007).
- Ivanova NV, deWaard JR, Hebert PDN (2006). An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes* **6**: 998-1002.
- Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN (2007). Universal primer cocktails for fish DNA barcoding. *Molecular Ecology Notes*, **7**, 544-548.
- Marko PB, Lee SC, Rice AM, Gramling JM, Fitzhenry TM, McAlister JS, Harper GR, Moran AL (2004). Mislabeling of a depleted reef fish. *Nature* **430**: 309-310.
- Messing J (1983) New M13 vectors for cloning. *Methods in Enzymology* **101**: 20-78.
- Palumbi SR (1996). Nucleic acids II: The polymerase chain reaction. pp 205-247 In: *Molecular Systematics*, eds Hillis DM, Moritz C, Mable BK, Sinauer Associates Inc., Sunderland.
- Ratnasingham S, Hebert PDN (2007). BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes* **7**: 355-364.
- Regulatory Fish Encyclopedia (RFE) Isoelectric Focusing (IEF) Peak Report (PKRPT) (2000), <http://www.cfsan.fda.gov/~frf/rfe5prsm.html>, accessed on October 11, 2007.

- Tenge, B.J, N.L. Dang, F.S. Fry, W.E. Savary, P.L. Rogers, J.D. Barnett, W.E. Hill, J.E. Wiskerchen, and M.M. Wekell. (1997). "Integration of Computer and Laboratory Techniques for Species Identification Including Development of a Regulatory Fish Encyclopedia" in Fish Inspection, Quality Control, and HACCP - A Global Focus, p. 214-226. Martin, Collette, and Slavin, eds., Technomic Publishing, Lancaster, PA.
- Yancy HF, Zemplak TS, Mason JA, Washington JD, Tenge BJ, Nguyen NT, Barnett JD, Savary WE, Hill WE, Moore MM, Fry FS, Randolph SC, Rogers PL and Hebert PDN (2008). The Potential Use of DNA Barcodes in Regulatory Science: Applications of the Regulatory Fish Encyclopedia. *Journal of Food Protection* **71**(1):210-7.

CONFIDENTIAL

Appendix

Acceptable Methods* for Tissue Lysis and DNA Extraction

- (I) DNeasy Blood & Tissue Kit Qiagen Catalog No. 69504 (50) or 69506 (250) (U. Guelph)
- (II) Automated DNA extraction on Beckman/Coulter Biomek FX (U. Guelph)
- (III) BioSprint 96 DNA Blood Kit (Qiagen) (Smithsonian Inst.)
- (IV) AutoGenPrep 965 (Smithsonian Inst.)

*Additional published and commercial methods are available but would need to be evaluated separately to show that they yield quality DNA suitable for PCR and sequencing.

(I) DNeasy Blood & Tissue Kit Qiagen Catalog No. 69504 (50) or 69506 (250) (Method 1)

Reagents

- a. Collection Tubes (2 ml) 100 500
- b. Buffer ATL 10 ml 50 ml
- c. Buffer AL* 12 ml 54 ml
- d. Buffer AW1 (concentrate) 19 ml 95 ml
- e. Buffer AW2 (concentrate) 13 ml 66 ml
- f. Buffer AE 22 ml 2 x 60 ml
- g. Proteinase K 1.25 ml 6 ml

Consumables & Equipment

- a. Pipets and pipet tips
- b. Vortexer
- c. Ethanol (96-100%)
- d. Optional: RNase A (100 mg/ml) Qiagen, Catalog No. 19101
- e. Microcentrifuge tubes (1.5 ml or 2.0 ml)
- f. Microcentrifuge with rotor for 1.5 ml and 2.0 ml tubes
- g. Thermomixer, shaking water bath. Or rocking platform for heating at 56°C

Preparation of Buffer AW1, AW2, and AL

- a. Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate volume of ethanol (96–100%) as indicated on the bottle and shake thoroughly. Buffer AW1 and Buffer AW2 are stable for at least 1 year after the addition of ethanol when stored closed at room temperature (15–25°C).
- b. Buffer AL and ethanol (96–100%) are added in the same step. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples.

- c. Add 90 ml ethanol (96–100%) to the bottle containing 86 ml Buffer AL or 260 ml ethanol to the bottle containing 247 ml Buffer AL and shake thoroughly. Mark the bottle to indicate that ethanol has been added. (Please note that, for purification of DNA from animal blood, Buffer AL must be used without ethanol. Buffer AL can be purchased separately if the same kit will be used for purification of DNA from animal blood.) Buffer AL is stable for 1 year after the addition of ethanol when stored closed at room temperature.

Important points before starting

- a. If using the DNeasy Blood & Tissue Kit for the first time, read “Important Notes” (Handbook, page 15 [available at: <http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000641>])
- b. For fixed tissues, refer to the pretreatment protocols “Pretreatment for Paraffin-Embedded Tissue”, page 41, and “Pretreatment for Formalin-Fixed Tissue”, page 43.
- c. All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.

Things to do before starting

- a. Buffer ATL and Buffer AL may form precipitates upon storage. If necessary, warm to 56°C until the precipitates have fully dissolved.
- b. Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- c. Preheat a thermomixer, shaking water bath, or rocking platform to 56°C for use in step 2.
- d. If using frozen tissue, equilibrate the sample to room temperature. Avoid repeated thawing and freezing of samples since this will lead to reduced DNA size.

Protocols for Tissue Lysis and DNA Extraction

- a. Cut up to 25 mg tissue into small pieces, and place in a 1.5 ml microcentrifuge tube. Note: It is strongly recommend cutting the tissue into small pieces to enable more efficient lysis.
- b. Add 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking platform. Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. If it is more convenient, samples can be lysed overnight; this will not affect them adversely. After incubation the lysate may appear viscous, but should not be gelatinous as it may clog the DNeasy Mini spin column. If the lysate appears very gelatinous, see the “Troubleshooting Guide”, page 47, in DNeasy Blood & Tissue Kit handbook for recommendations.
- c. Vortex for 15 s. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing. It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous

solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples. **Note:** A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure.

- d. Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.
- e. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at 6000 x g (8000 rpm). Discard flow-through and collection tube.
- f. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube. **Note:** It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).
- g. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 100 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute.
- h. Recommended: For maximum DNA yield, repeat elution once as described in step g. **Note:** A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step g can be reused for the second elution step. Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

(II) Automated DNA Extraction on Beckman/Coulter Biomek FX (Method 2)

Reagents

Note: Abbreviation indicated in parentheses

- a. Disodium ethylenediamine tetra-acetate x 2H₂O (EDTA); Fisher Scientific Catalog No. S311-500
- b. ELIMINase; Decon Labs Inc. 1102
- c. Reagent Alcohol, Histological. (EtOH 96%); Fisher Scientific Catalog No. A962-4
- d. Guanidine thiocyanate (GuSCN); Fisher Scientific Catalog No. BP 166-500
- e. Molecular grade water (dd H₂O); Invitrogen Catalog No. 10977023

- f. Polyethylene glycol sorbitan monolaurate (Tween-20); Fisher Scientific Catalog No. ICN1947245
- g. Proteinase K; Invitrogen Catalog No. 25530031
- h. Sodium chloride (NaCl); Fisher Scientific Catalog No. S271-3
- i. Sodium dodecyl sulfate (SDS); GibcoBRL Catalog No. 15525-025
- j. Sodium hydroxide (NaOH); Fisher Scientific Catalog No. S318-3
- k. t-Octylphenoxypolyethoxyethanol (Triton X-100); Fisher Scientific Catalog No. ICN807426
- l. Tris(hydroxymethyl)aminomethane (Trizma base); Sigma Catalog No. T6066-100g
- m. Tris(hydroxymethyl)aminomethane hydrochloride (Trizma HCl); Catalog No. Sigma T5941-100g
- n. Glycerol; Sigma Catalog No. G5516-500ml
- o. Hydrochloric Acid (HCl); Fisher Scientific Catalog No. AC124210010

Consumables & Equipment

- a. ABGene 8-Strip flat PCR caps (cap strips); Fisher Catalog No. AB-0783
- b. AcroPrep 96 1 ml filter plate with 1.0 μm Glass Fiber media, natural housing (PALL plate); PALL Catalog No. 5051
- c. Matrix Impact2 pipettor, 15 μl -1250 μl (8-channel Matrix multichannel pipette); Matrix Technologies Catalog No. 2004
- d. Matrix Technologies 1250 μl Pipet Tip (Non-filter tip); Matrix Technologies Catalog No. 8046
- e. Matrix Technologies 1250 μL Talltip (102mm); (Filter tip); Matrix Catalog No. 8245
- f. PP MASTERBLOCK, 96 Well, 2 ml (square-well block); Greiner Catalog No. 780271
- g. SBS Receiver Plate Collar (PALL collar); PALL Catalog No. 5225
- h. Nalgene filter unit 0.2 μm membrane; Nalgene Catalog No. 450-0020
- i. Eppendorf Research Single Channel Pipetter (200-1000 μl); Fisher Scientific Catalog No. 13-688-514
- j. Eppendorf twin-tec 96 PCR plate; Fisher Scientific Catalog No. E951020460
- k. Span-8 P250 non sterile tips; Beckman Coulter Catalog No. CABK379501
- l. Span-8 P250 sterile barrier tips; Beckman Coulter Catalog No. CABK379503
- m. Span-8 P20 sterile barrier tips; Beckman Coulter Catalog No. CABK379506
- n. Reagent reservoirs; VWR Catalog No. 82026-352
- o. Biomek® FXp Laboratory Automated Workstation; Beckman Coulter Catalog No. A31844
- p. Simplicity® Water Purification System; Millipore Corporation. Fisher Scientific Catalog No. SIMSV00US
- q. Allegra ® 25R Refrigerated Centrifuge; Beckman Coulter Catalog No. 369434
- r. Fisher Isotemp Incubator; Fisher Scientific Catalog No. 11-690-637D
- s. Innova Platform Shaker; Fisher Scientific Catalog No. 14-278-105.
- t. Orion Star® pH Meter; Thermo Electron Corporation. Catalog No. 1111000

Stock Solutions

Note: Thoroughly wash all labware with ELIMINase and then rinse with dH₂O. Weigh reagents using a clean spatula. Use molecular grade doubly distilled (dd) H₂O in all buffer formulations.

Filter buffers through 0.2 μm Nalgene filter into a clean sterile bottle; make smaller volume working aliquots (e.g. 100 ml). Store stock solutions and working aliquots at 4°C.

- a. 1M Tris-HCl, pH 8.0 (26.5 g Trizma base and 44.4 g Trizma HCl/500 ml water)
- b. 1M Tris-HCl, pH 7.4 (9.7 g Trizma base, 66.1 g Trizma HCl/500 ml water)
- c. 0.1M Tris-HCl, pH 6.4 (6.06 g Trizma base/500 ml water). Note: adjust pH with HCl to 6.4-6.5 before adding water to a final volume.
- d. 1M NaCl (29.22 g NaCl/500 ml water)
- e. 1N NaOH (20 g NaOH/500 ml water)
- f. 0.5 M EDTA pH 8.0 (186.1 g EDTA, ~20.0 g NaOH/1000 ml water).

Note: Vigorously mix on magnetic stirrer with heater. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH. Give a brief rinse to NaOH granules with dd H₂O in a separate glass before dissolving them. Adjust pH to 8.0 with 1N NaOH, before bringing to a final volume).

- g. Proteinase K (20 mg/ml) in 10 mM Tris-HCl, pH 7.4, 50% glycerol v/v
Note: Add 20 ml of water and 0.5 ml of 1M Tris-HCl, pH 7.4 to a vial with 1 g of Proteinase K, close the lid, mix well by inverting, do not shake. Pour into graduated cylinder, add water to 25 ml, then add 25 ml of glycerol, mix well on magnetic stirrer. Do not filter.

Working Solutions for DNA Extraction

Note: Weigh the dry components (e.g. SDS or GuSCN) first, then add required volumes of the stock solutions using a graduated cylinder or serological pipette, and add molecular grade dd H₂O to the final volume. No filtering is required.

- a. Vertebrate Lysis Buffer (VLB): 100 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA, pH 8.0, 0.5% SDS (20 ml 1M NaCl, 10 ml 1M Tris-HCl, pH 8.0, 1 g SDS/to 200 ml with water).
- b. Binding Buffer (BB): 6M GuSCN, 20 mM EDTA pH 8.0, 10 mM Tris-HCl pH 6.4, 4% Triton X-100 (354.6 g GuSCN, 20 ml 0.5M EDTA pH.8.0, 50 ml 0.1M Tris-HCl pH 6.4, 20 ml Triton X-100/to 500 ml with water). Store at room temperature for 1 month, dissolve at 56°C before use if any crystallization occurs.
- c. Wash Buffer (WB): 60 % EtOH, 50 mM NaCl, 10 mM Tris-HCl pH 7.4, 0.5 mM EDTA pH 8.0 (600 ml EtOH, 23.75 ml 1M NaCl, 9.5 ml 1M Tris-HCl, pH 7.4, 0.950 ml 0.5M EDTA, pH 8.0/to 950 ml with water). Store at -20°C.
- d. Binding Mix (BM): 250 ml Binding Buffer, 250 ml EtOH.
- e. Protein Wash Buffer (PWB): 260 ml Binding Buffer, 700 ml EtOH, 40 ml water.

Protocols for Tissue Lysis and DNA Extraction

Lysis

- a. Mix 5 ml of Vertebrate Lysis Buffer and 0.5 ml of Proteinase K, 20 mg/ml in a sterile container. Add 50 μl of this Lysis Mix to each well of a 96-well Eppendorf plate.

- b. Add a small amount of tissue (e.g. 1-2 mm³ of ethanol preserved tissue) to each well of the same 96-well solid skirted microplate (Wipe instruments with ETOH between samples). Cover plate with cap strips.
- c. Incubate at 56 °C for a minimum of 12 hours to allow digestion.
- d. Centrifuge at 1500 g for 15 sec in an Allegra 25 to remove any condensate from the cap strips.

Maintenance and check before running robotic DNA extraction

- a. Wipe any spills off the Beckman FX deck with a clean Kimwipe and de-ionized water.
- b. Check manifold gasket and wipe with de-ionized water if necessary.
- c. Check the manifold waste bottle; empty if necessary.
- d. Fill water supply bucket (cabinet on the left) for a wash station with 6 l of de-ionized water.
- e. Check wash station waste bucket (cabinet on the right); empty if necessary.
- f. Remove the covers from refillable reservoirs.
- g. Pour fresh buffers into bottles (for 10 plates ~ 300 ml BB, 600 ml PWB, 950 ml WB).
- h. Open vent holes on the buffer bottles.
- i. Start peristaltic pumps for the reservoirs and wash station – Instrument – Manual Control – Device Controller – Click on Simple1, Simple2, Simple3 and WashStation1.
- j. Let run for about 5 min, then unclick each of them.
- k. Click on Instrument setup, arrange plates on the deck.

Automated DNA extraction on Biomek FX

- a. In the Biomek FX Software, open file 'DNA_extraction_deck_2plates'. Load the deck of liquid-handling unit with labware and reagents according to the instrument setup layout. Turn ON the vacuum pump.
- b. Start the method. On the prompt confirm that the vacuum pump is turned ON. Steps c-p are performed automatically.
- c. Move GF1 plate to SPE (on top of vacuum manifold).
- d. Add 100 µl of BM to each well in L1 plate.
- e. Mix each lysate by repeatedly (4X) withdrawing and re-injecting 50 µl. Transfer 125 µl of each lysate into a GF1 plate sitting on a 36 mm collar positioned on the vacuum manifold. Apply a vacuum of 23 In Hg for 1 min and discard the filtrate.
- f. Return LT1 tips to their home destination.
- g. Load PWB tips.
- h. Add 180 µl of PWB to each well of BINDING1 and apply vacuum for 2 min.
- i. For the first wash step, add 220 µl of WB to each well and apply vacuum for 1 min.
- j. Wash PWB tips in a Wash Station unload PWB tips, move them to their home destination.
- k. Load WB tips.
- l. For the second wash step, add 660 µl of WB to each well and apply vacuum for 3 min to dry the plate; pause SPE for 5 min.
- m. Wash WB tips in a Wash Station unload WB tips, move them to their home destination.
- n. Pause SPE for 5 min to release the vacuum.
- o. Move GF1 plate to its home destination

- p. Move GF2 plate to SPE.
 - q. Repeat steps 4-15 (use new tips LT2 for lysate transfer).
 - r. After the method is completed, remove the plates manually, place them on top of collection plates, cover the top with clear film, place assembly on top of square well block and centrifuge at 5000 g for 2 min. Shake off any collected wash buffer, and incubate at 56°C for 30 min to evaporate residual ethanol. Separate plates before putting in the incubator, flip filter plate on a long side to make sure that tips don't touch any surface in the incubator.
 - s. Place the plates back on top of 96-well microplates preassembled with a PALL collar.
 - t. Add 50 µl of dd H₂O preheated to 56°C to each well, cover the top with clear film and centrifuge at 5000 g for 5 min.
 - u. Remove filter plate and collar and cover collection plate with aluminum foil.
 - v. Store the microplate with the DNA extracts at 4°C.
-

(III) BioSprint 96 DNA Blood Kit (Qiagen) (Method 3)

Equipment and Reagents (Qiagen Catalog numbers)

- a. BioSprint 96 workstation, cat. No. 9000852
- b. Magnetic head for use with large 96-rod covers (supplied with BioSprint 96)
- c. BioSprint 96 DNA Blood Kit (48), cat. No. 940054, or BioSprint 96 DNA Blood Kit (384), cat. No. 940057
- d. Microcentrifuge or centrifuge capable of holding S-blocks (e.g., Centrifuge 4-15C)
- e. Buffer ATL, cat. No. 19076
- f. QIAGEN Proteinase K (2 ml), cat. No. 19131, or QIAGEN Proteinase K (10 ml), cat. No. 19133
- g. Tape Pads (5), cat. No. 19570
- h. Optional: DNase-free RNase A (required if purified DNA needs to be RNA-free)
- i. Multichannel pipettor and disposable pipet tips with aerosol barriers (20-1000 µl)
- j. Ethanol (96-100%)
- k. Isopropanol (100%)
- l. Vortexer
- m. Shaking incubator (e.g., Thermomixer from Eppendorf)
- n. Tween -20

Preparations

Preparation of buffer AW1, AW2, and AL

- a. Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate volume of ethanol (96-100%) as indicated on the bottle and shake thoroughly. Buffer AW1 and Buffer AW2 are stable for at least 1 year after the addition of ethanol when stored closed at room temperature (15-25°C).

- b. Buffer AL, isopropanol (96-100%) and magnetic beads are added in the same step. Buffer AL, isopropanol (96-100%) and magnetic beads can be premixed and added together in one step to save time when processing multiple samples.

Important notes before starting

- a. Check that Buffer AW1, Buffer AW2, and RNase-free water have been prepared according to the instructions in the “Preparing reagents” section of the *BioSprint DNA Blood Handbook*. [available at: http://www.thermo.com/eThermo/CMA/PDFs/Various/File_28875.pdf]
- b. Check that Buffers AL and ATL do not contain a white precipitate. If necessary, incubate both for 30 minutes at 37°C with occasional shaking to dissolve precipitate.
- c. Ensure that you are familiar with operating the BioSprint 96. Refer to the *BioSprint 96 User Manual* for operating instructions.
- d. 96-rod covers are supplied either as packets of two, or as packets of one inserted into an S-block. If using a new packet of two, store the second 96 rod cover on another plate. It is important that the 96-rod cover does not become bent.

Things to do before starting

- a. Set a shaking incubator with a 96-well plate adapter to 56°C for use in step 2.
- b. MagAttract magnetic particles copurify RNA and DNA if both are present in the sample. IF RNA-free DNA is required, add RNase A to the sample before starting the procedure. The final RNase A concentration should be 2 mg/ml (e.g., add 4 µl of a 100 mg/ml RNase A solution to a 200 µl sample).

Prepare a master mix

- a. Prepare a master mix 10% greater than that required for the total number of sample purifications to be performed by mixing 200 µl of Buffer AL, 200 µl of Isopropanol (100%) and 30 µl MagAttract Suspension G per reaction. Before adding MagAttract Suspension G, ensure that it is fully resuspended. Vortex for 3 min before using for the first time, and for 1 min before subsequent uses.

Protocols for Tissue Lysis and DNA Extraction

Lysis

- a. Cut each animal tissue sample into small pieces. Place tissue samples into the wells of an S-block or into 1.5 ml microcentrifuge tubes, and add 180 µl Buffer ATL.
- b. Add 20 µl proteinase K, seal the S-block with an Axygen silicone mat held down with a hard plastic cover that is taped on or close the microcentrifuge tubes, and incubate the samples at 56°C in a shaking incubator until the tissue is completely lysed. Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1-3 h. If it is more convenient, samples can be lysed overnight; this will not affect the DNA quality. Optional: If RNA-free genomic DNA is required, add 4 µl of RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature (15-25°C).
- c. Towards the end of the proteinase K digestion, begin preparation of 5 S-blocks (slots 2-6) and 96-well microplates (slots 7 and 8) as detailed in the next section.

Preparation of S-blocks and 96-well microplates

In each plate or block, the number of wells to be filled with buffer should match the number of samples to be processed (e.g., if processing 48 samples, fill 48 wells per plate or block). Ensure that buffers are added to the same positions in each plate or block (e.g., if processing 48 samples, fill wells A1-H1 to A6-H6 of each plate or block).

- a. For slot 2 S-block, add 650 μ l of Buffer AW1 per well. The message when loading should read “Load Wash 1”.
- b. For slot 3 S-block, add 500 μ l of Buffer AW1 per well. The message when loading should read “Load Wash 2”.
- c. For slot 4 S-block, add 500 μ l of Buffer AW2 per well. The message when loading should read “Load Wash 3.”
- d. For slot 5 S-block, add 500 μ l of Buffer AW2 per well. The message when loading should read “Load Wash 4”.
- e. For slot 6 S-block, add 500 μ l RNase-free water, which contains 0.02%(v/v) Tween-20, per well. The message when loading should read “Load Wash 5”.
- f. For slot 7 96-well microplate MP, add 200 μ l Buffer AE per well. The message when loading should read “Load Elution”.
- g. For slot 8 96-well microplate MP, load the large 96-rod cover. The message when loading should read “Load Rod Cover.”

DNA Extraction

- a. Briefly centrifuge the S-block or microcentrifuge tubes containing the sample to remove drops from underneath the silicone mat or inside the lids. Remove the mat from the S-block.
- b. Vortex the master mix containing Buffer AL, isopropanol, and MagAttract Suspension G for 1 min. Add 430 μ l of master mix to each sample in the S-block. Note: If using an Eppendorf Multipipette, aliquot 450 μ l master mix to each sample. The starting volume of master mix should be increased accordingly.
- c. Switch on the BioSprint 96 at the power switch.
- d. Slide open the front door of the protective cover.
- e. Select the protocol “BS96 DNA tissue” using the up and down arrow keys on the BioSprint 96 workstation. Press “Start” to start the protocol run.
- f. The LCD displays a message asking you to load slot 8 of the worktable with the 96-rod cover. After loading 8, press “Start”. The worktable rotates and a new message appears, asking you to load slot 7 with the elution plate. Load slot 7 and press “Start” again. Continue this process of pressing “Start” and loading a particular slot until all slots are loaded. Note: Each slot is labeled with a number. Load each 96-well plate or S-block so that well AI is aligned with the slot’s label (i.e., well AI faces inward).
- g. Check that the protective cover is correctly installed: it should fit exactly into the body of the BioSprint 96. Slide the door shut to protect samples from contamination. Warning: To avoid contact with moving parts during operation of the BioSprint 96, do not insert your hands and fingers inside the work station.
- h. Press “Start” to start sample processing.

- i. After the samples are processed, remove the plates and blocks as instructed by the display of the BioSprint 96. Press “Start” after removing each plate or block. The first item to be removed contains the purified samples. Carryover of magnetic particles in elutes will not affect most downstream applications. If the risk of magnetic-particle carryover needs to be minimized, the microplate containing eluates should first be placed in a suitable magnet and the eluates transferred to a clean microplate (see the appendix, in the *BioSprint DNA Blood Handbook*).
 - j. Press “Stop” after all plates and blocks are removed.
 - k. Discard the used plates, blocks, and 96-rod cover according to your local safety regulations. Note: See “Safety Information” in the *BioSprint DNA Blood Handbook*. We collect plate 1, 2 and 3 which contains AL and AW1 with guanidinium chloride as hazardous waste.
 - l. Switch off the BioSprint 96 at the power switch.
 - m. Wipe the worktable and adjacent surfaces using a soft cloth or tissue moistened with distilled water or detergent solution. If infectious material is spilt on the worktable, clean using 70% ethanol or other disinfectant. Note: Do not use bleach as disinfectant. See “Safety Information” in the *BioSprint DNA Blood Handbook*.
-

(IV) AutoGenprep 965 (AutoGen) Reagents and Equipment (Method 4)

Reagents and Equipment

- a. Reagent M1
- b. Reagent M2
- c. Proteinase K
- d. Reagent R3
- e. Reagent R4
- f. Reagent R5
- g. Reagent R6
- h. Reagent R7
- i. Reagent R8
- j. Reagent R9

Preparations

Preparation of solution

- a. Prepare solution for every run by dissolving appropriate aliquots of Proteinase K provided in 965/960 kit with each aliquot of Reagent M1. Standard concentration for overnight digestion of animal tissue is 0.4 mg/ml. For other types of tissues or quicker digestion, a higher concentration of Proteinase K solution may be required.

Protocols for Tissue Lysis and DNA Extraction

Lysis Protocol A

- a. Place 0.5-1.0 cm animal tissue (10-20mg) into 96 deep well plate(s) from Costar (#3960).
- b. Load the sample plates on the AutoGenprep 965/960
- c. Run [Digest] protocol. The AutoGenprep 965/960 adds 0.15 ml of Reagent M2 (Tissue Digestion 2) and 0.15 ml of Reagent M1 (Tissue Digestion 1), containing the pre-dissolved Proteinase K at the concentration of 0.4-1.0mg/ml into the plates and mixes them.
- d. Remove the sample plate(s) from the 965/960, seal the plate and incubate overnight at 56 °C.

Lysis Protocol B

- a. Place animal tissue into 96 deep well plate(s) {Costar (#3960)} or appropriate tubes, and add equal amounts (150 µl) of Reagent M2 (Tissue Digestion 2) and Reagent M1 (Tissue Digestion 1), containing the pre-dissolved Proteinase K at the concentration of 0.4-1.0 mg/ml for a final concentration of 15-60 mg tissue/ml.
- b. Incubate the samples overnight at 56°C.
- c. Transfer 0.3 ml (equivalent to 5-20mg tissue) of Proteinase K digests to 96 well deep well plates.

DNA Extraction

- a. Add 0.3 ml of Proteinase K digest (equivalent to 5-20mg of tissues) samples to a maximum of 384 samples (4 plates) per run. There is a minimum of 2 plates and 24 samples (12 samples of Row A in each plate) for the centrifuge to be balanced properly.
- b. Load the AutoGenprep 965/960 with sample plates, equal number of output plates and tips. Tips include one box for the reagents and a box for each sample plate.
- c. Load Reagents R3, R4, R5/R6/R7, R8 and R9 into the appropriate reservoirs, i.e. R3 goes in reservoir 3. Note – reservoir 7 is an optional 3rd wash step and is not in the standard run, so does not need to be filled with reagent.
- d. Turn on computer and the AutoGenprep 965/960. Once turned on, open the AutoGenprep 965 software.
- e. Select [Extraction] protocol.
- f. Enter number of samples. Samples are in increments of 24.
- g. Set the resuspension volume (Reagent R9) to 0.1 ml if desired. Standard is 0.05 ml
- h. Start the run.

Run consist of:

- a. Add Reagent R8 and mix.
- b. Add Reagent R3, mix, centrifuge to pellet debris and transfer supernatant to new (DNA) plate.
- c. Add Reagent R4, mix, precipitate DNA, centrifuge and discard supernatant.
- d. Add Reagents R5/R6/R7, mix, centrifuge, and discard supernatant, repeat.

- e. Dry DNA by blowing air across the top of plates.
- f. Add Reagent R9 and mix to resuspend DNA.

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