CENTER FOR MOLECULAR DYNAMICS - NEPAL

DNA Barcoding of fish specimen preserved in Formalin

To:

Fisheries Research Department (FRD)

FINAL REPORT

Report by:
CMDN

Contents

Among our outlined project goals signed between CMDN and USAID funded PAANI project, one of the listed objective was to develop genetic profile of the fish specimens stored in formalin at Fisheries Research Department, Godavari.

Sample Collection:

A team of five researchers (Lab team/ field team/ Fish morphological expert) visited FRD to dissect tissue (caudal peduncle) from the museum stored samples.

Researchers on board during Sample collection:

- Adarsh Man Sherchan (Senior Operational Manager)
- Jyoti Joshi (Genetics Program Manager/Senior Research Associate)
- Hemanta Kumari Chaudhary (Senior Research Associate)
- Samita Raut (Field Researcher)
- Anjana Shrestha (Fish Morphology Expert)

Fig 1. Museum Speciem stored in Formalin at FDR

Fig 2. FDR staff helping CMDN fish team for sorting museum specimens

Fig: 3 CMDN fish team dissecting the selected fish specimen

DNA extraction and Amplification

A set of different extraction protocols were implemented to extract high yield DNA from the formalin stored samples. However, isolating high-quality genomic DNA from formalin-fixed PET can be difficult because only minimal amounts of intact DNA may be present in the sample.

It is thought that exposure of tissue nucleoproteins to formalin results in the formation of cross-links between protein and DNA complexes (Lin et.al 2002). Formalin (4% formaldehyde) cross-links both histones to histones and histones to DNA. The cross-linking of biomolecules to DNA could directly reduce the PCR amplification

efficiency from the genomic DNA of formalin-fixed tissue. Crosslinked DNA-biomolecules may also prevent the complete digestion of proteins by proteinase K of the formalin- fixed tissues before the extraction of genomic DNA and therefore significantly decrease the yield of amplifiable genomic DNA.

PROTOCOLS USED FOR EXTRACTING DNA:

Gene All- Tissue extraction Kit

We extracted DNA using Gene All-Tissue extraction Kit following the user manual.
Extracted DNA was used as a template for PCR amplification of two gene marker CO1 and 12S.
The partial COI segment of mt DNA was targeted for DNA barcoding using cocktail mix of fish specific primers (Table 1) which amplified ~650 bp region of the gene (Ivanova et. al, 2007).

Table 1: Primers used for COI amplification

Target gene	Primers ID	Sequences(5' - 3')	Band size(bp)	Reference
COI	VF2_t1	TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC	~ 650bp	(Ivanova et al., 2007)
	FishF2_t1	TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC		
	FishR2_t1	CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA		
	FR1d_t1	CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA		
	M13F(-21)	TGTAAAACGACGGCCAGT		
	M13R (-27)	CAGGAAACAGCTATGAC		

A total of 25µL PCR final reaction was prepared containing 12.5 µL of 2X Qiagen multiplex master mixes, 2.5 µL of 5X Q-solution, 0.25 µL 10pMol/ µLFish COI Cocktail primer sets and 2 µL of extracted DNA. The thermocycling (MJ Research Tetrad PTC-225 Thermal Cycler, USA) condition was 95 ºC for 15 min followed by touch down PCR of 5 cycles of 94 ºC for 60 sec, 48 ºC for 50 sec and 72 º C for 50 sec followed by 35 cycles of

94 ºC for 60 sec, 50 ºC for 50 sec and 72 º C for 50 sec with the final extension at 72 º C for 5 min. Amplified ~ 650 bp target PCR product was visualized on Gel-Doc, (Major ScientificTM) under 2% agarose gel electrophoresis.

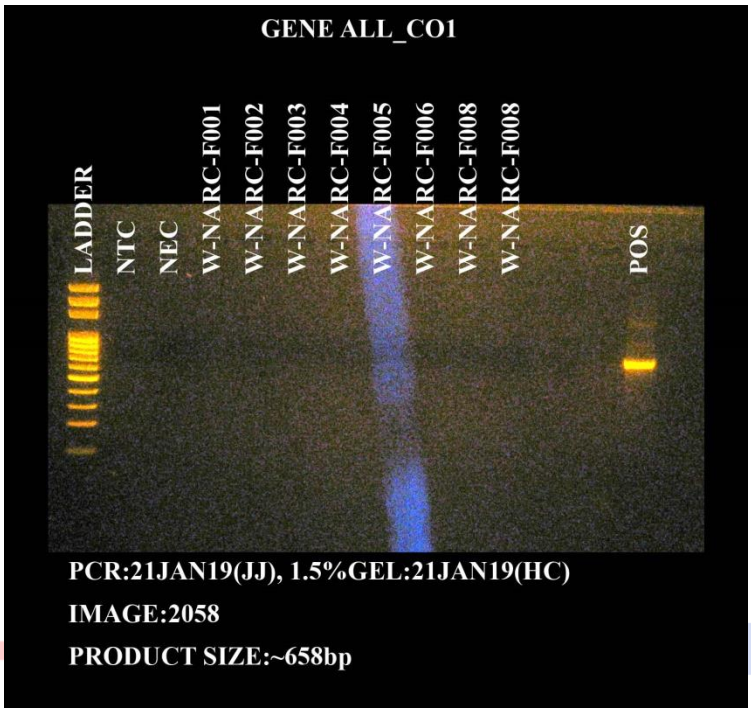

Fig 4. Cytochrome Oxidase-I PCR run on 1.5% agarose gel with bands appearing at approximately (650) bp for known positive.

DNA extracted from Gene All Tissue kit failed to amplify CO1 gene (~650bp) (no bands for samples in the gel image above). Extraction negative control (NEC) and PCR negative control (NTC) are appearing negative thus, showing no contamination in the DNA isolation and amplification methods.

For the amplification of 12S gene following primer sets were used.

Table 2: Primers used for 12S amplification

Target gene	Primers ID	Sequences(5' - 3')	Band size(bp)	Reference
12S	MiFish-U-F	GTCGGTAAAACTCGTGCCAGC	~ 180bp	*(Miya et al., 2015)*
	MiFish-U-R	CATAGTGGGGTATCTAATCCCAGTTTG		

For 12S Gene fragment amplification, a total of 25 µL PCR final reaction was prepared containing 12.5 µL of 2 X Qiagen multiplex master mixes, 2.5 µL of 5X Q-solution, each 1.25 µL of 10pMol/ µLMiFish-U-F and MiFish-U-R primer sets and 2 µL of extracted DNA. The thermocycling (MJ Research Tetrad PTC-225 Thermal Cycler, USA) condition was 95 ºC for 15 min followed by cycles of 30, 98 ºC for 5sec, 50 ºC for 10 sec and 72 ºC for 10 sec with the final extension at 72 ºC for 5 min. Amplified ~ 200bp target PCR product was visualized on Gel-Doc, (Major Scientific™) under 2% agarose gel electrophoresis.

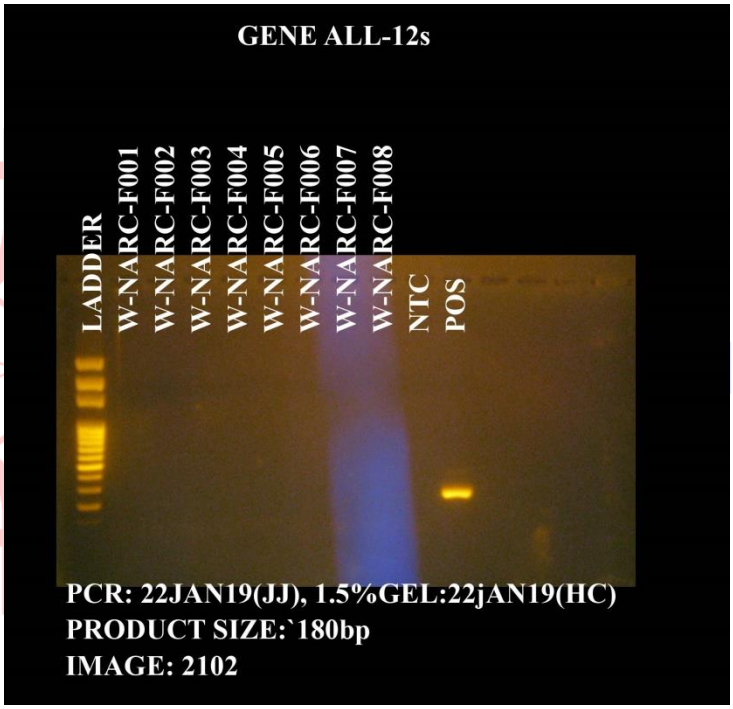

Fig 5.12S PCR run on 1.5% agarose gel with positive bands appearing at approximately (180) bp

Result: DNA extracted from Gene All Tissue kit failed to amplify 12s gene (~180bp) (no bands for samples in the gel image above).

ZYMO Research FFPE KIT

KIT PROTOCOL:

Tissue Digestion

To the deparaffinized tissue sample (≤ 25 mg) in a microcentrifuge tube, add the following mixture:

H2O 45µl, 2X Digestion Buffer, 45µlProteinase K 10µl

Rapid Digestion Standard Digestion

Incubate at 55°C for 1-4 hours Incubate at 55°C overnight (12-16 hrs)

Transfer the digestion to 94°C and incubate for 20 minutes.

DNA Purification

1. Add 350 µl of Genomic Lysis Buffer to the tube and mix thoroughly by vortexing.

2. Add 135 µl of isopropanol1 (user supplied) to the sample and mix thoroughly.

Centrifuge at ≥ 12,000 x g for 1 minute to remove insoluble debris.

3. Transfer the supernatant to a Zymo-Spin™ IIC Column2 in a Collection Tube.

Centrifuge at 10,000 x g for 1 minute.

4. Add 400 µl of Genomic DNA Wash 1 to the spin column in a new Collection Tube. Centrifuge at 10,000 x g for 1 minute. Discard the flow-through.

5. Add 700 µl of Genomic DNA Wash 2 to the spin column. Centrifuge at ≥ 12,000 x g for 1 minute. Discard the flow-through.

6. Add 200 µl of Genomic DNA Wash 2 to the spin column. Centrifuge at ≥ 12,000 x g for 1 minute.

7. Transfer the Zymo-Spin™ IIC Column to a clean microcentrifuge tube. Add ≥ 50 µl DNA Elution Buffer3 or water (add ≥100 µl if sampling 25 mg tissue) to the spin column. Incubate 2-5 minutes at room temperature.

8. Centrifuge at top speed for 30 seconds to elute the DNA.

The eluted DNA can be used immediately for molecular based applications or stored ≤-20ºC for future use.

Result:

DNA extracted using ZYMO FFPE kit was used as a template to amplify 12S gene. (Fig 6)

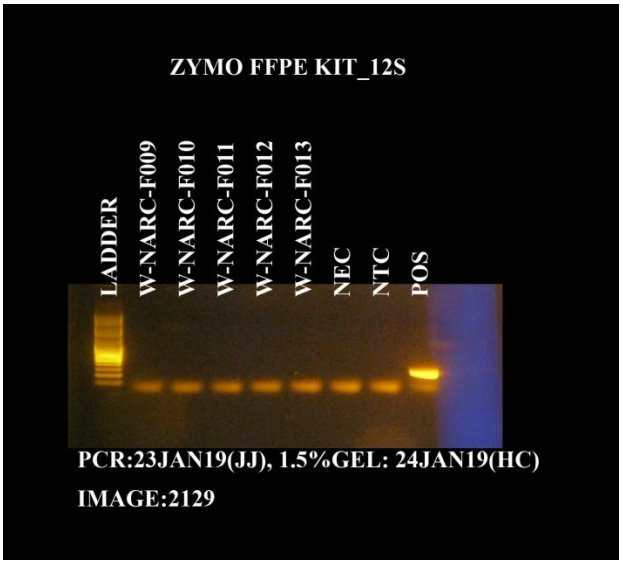

Fig 6.12S PCR run on 1.5% agarose gel with positive bands appearing at approximately (180) bp

Hence, samples extracted with ZYMO FEPE kit failed to amplify.

Qiagen Modified Protocol

For the QIAamp DNA mini kit, 180UL of buffer ATL was added to a tissue sample in a 1.5-mL microcentrifuge tube. The microcentrifuge tube was then placed in a heating block set at 98°C for 15 minutes and briefly cooled at room temperature for 5 minutes. Proteinase K solution (20 ul) was added to the heat-treated tissue section. The tissue sample was incubated at 68°C for 45minutes. Buffer AL (200 ul) was added to the sample, followed by incubation for 10 minutes at 72°C. After incubation, 200 ul ethanol was added to the sample, and the mixture was transferred a QIAamp Spin Column. The genomic DNA was retained on the column and washed twice with 500 ul of wash buffer before eluting with 200 ul of 10 mmol/L Tris-HCl (pH 8.0) at room temperature.

Reference: Extraction and Amplification of DNA From Formalin-Fixed, Paraffin-Embedded Tissues
*Lin Wu, Ph.D., *Nancy Patten, B.S., †Carl T. Yamashiro, Ph.D., and †Buena Chui, B.S.

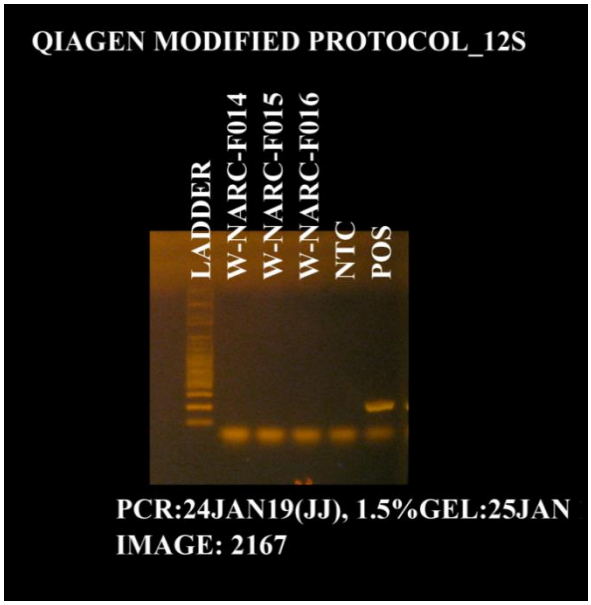

Fig 7.12S PCR run on 1.5% agarose gel with positive bands appearing at approximately (180)bp

Result: No amplified product as seen in the gel image above.

Extraction using Alkali treatment

Alkali digestion buffer: 0.1 M NaOH with 1% SDS solution.

Store at room temperature. The pH around 12.

Tissue Pre-Preparation

Tissue Digestion- Place tissue in 0.5 mL of the alkali digestion buffer in a 2-mL

tissue-buffer to cool for 5 min to room temperature

Proteinase K solution (20 ul) was added to the heat-treated tissue section. The tissue sample was incubated at 68°C for 45minutes.

After incubation, 200 ul ethanol was added to the sample, and the mixture was transferred a QIAamp Spin Column.

The genomic DNA was retained on the column and washed twice with 500 ul of wash buffer before eluting with 200 ul of 10 mmol/L Tris-HCl (pH 8.0) at room temperature

Reference: DNA Extraction from Formalin-Fixed Material

Paula F. Campos and Thomas M. P. Gilbert

Fig 8. 12S PCR run on 1.5% agarose gel with positive bands appearing at approximately (180) bp

Result: No amplified product as seen in the gel image above

PHIX 29 (TempliPhi Protocol)

TempliPhi DNA amplification kits are novel products developed specifically to prepare templates for DNA sequencing. TempliPhi method utilizes bacteriophage ø29 DNA polymerase to exponentially amplify single- or double-stranded circular DNA templates by rolling circle amplification (RCA). This isothermal amplification method produces microgram quantities of DNA from picogram amounts of starting material in a few hours.

Prepare the following master mix

Reagents	1X
10X Buffer	2ul

DD water	6ul
DNA	2ul
Total	10ul

Run Pretemp program on Thermal cycler **96°C for 3min**

Prepare mastermix and add in the previous products adding upto 20ul final volume

Reagents	1X
Phi 29	0.4ul
dNTPs	1ul
Random Primers	1ul
DD water	7.6ul
Total	10ul

Run templiphi program on thermal cycler (30°C for 16 hours and store at 4°C for down streaming process. We saw band above ladder while running only RCA product,

We used RCA product as a template for CO1 and 12S PCR.

Result: No amplification.

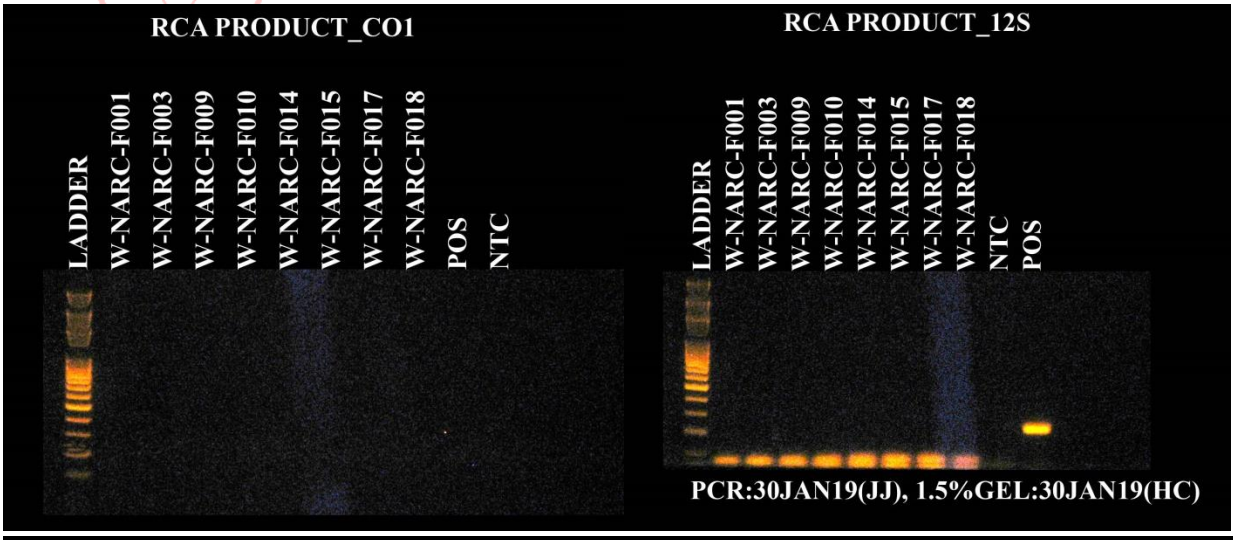

Fig 9. 12S PCR run on 1.5% agarose gel with positive bands appearing at approximately (180) bp

FRD provided two specimen for dissecting inner tissue

Two fish specimen was provided by FRD to dissect the inner organs of the fish because of lower to failed DNA yield from the tissue. We dipped the whole fish in ethanol overnight and dissected the tissue from brain, heart/liver and eye ball.

Dissected tissues were stored in ethanol overnight prior to extraction.

We extracted the DNA using alkali treatment followed by modified QIAGEN protocol (Prot k, AL buffer, ethanol, washing and elution) as stated above,

Extracted DNA was further amplified targeting 12S gene followed by gel electrophoresis.

Result: No amplification of the extracted DNA.

We used the extracted DNA for quantitation to determine the concentration in the extracted DNA using Qubit HS assay kit.

Table3. Quantification of extracted DNA using Qubit fluorometer

Sample Code	Quantity(ng/ul)
W-NARC-001	LOW
W-NARC-003	LOW
W-NARC-009	0.128
W-NARC-011	LOW
W-NARC-014	LOW
W-NARC-015	LOW
W-NARC-016	LOW

Phenol- Chloroform

We used Phenol-Chloroform as alternative method for extracting DNA from the formalin stored tissue samples.

We used samples stored in ethanol, formalin- dissected tissue stored in Longmire solution and NARC provided fish specimen (formalin stored) kept in ethanol after arrival in CMDN lab for comparison between the storage buffers.

We followed the protocol designed by Queen University.

Sample list:

- W-PAANI-F0049- ethanol stored
- W-PAANI-F0043- ethanol stored
- NARC BRAIN- formalin to ethanol shift
- NARC HEART- formalin to ethanol shift
- NARC LIVER- formalin to ethanol shift
- NARC CP- formalin to longmire
- NARC 20- formalin to longmire
- NARC 21- formalin to longmire

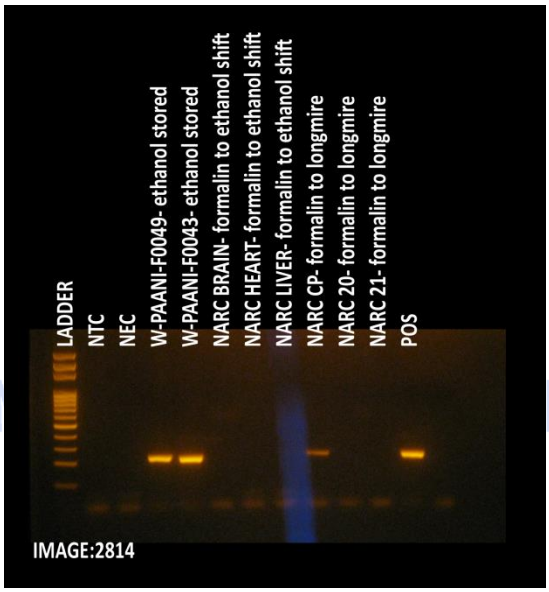

Fig 10. 12S PCR run on 1.5% agarose gel with positive bands appearing at approximately (180) bp

Result: Sample stored in ethanol amplified positive for 12S. One of the sample provided by Narc (transferred to ethanol, caudal peduncle dissected tissue) amplified using phenol-chloroform method. To check for cross contamination, we decided to re do extraction protocol on 10 of the formalin-longmire-ethanol transferred samples.

Re- extraction on 10 samples using Phenol Chloroform

We transferred the Longmire stored samples in ethanol prior to one day before the extraction day.

We followed the phenol-chloroform extraction as stated in section 7.

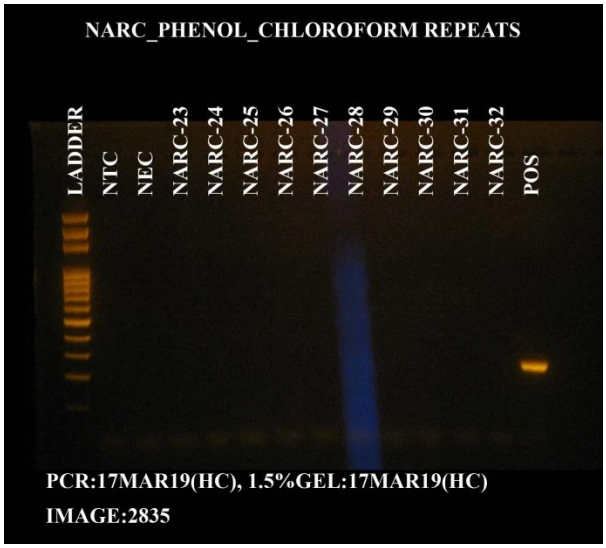

Fig 11. 12S PCR run on 1.5% agarose gel with positive bands appearing at approximately (180) bp

Result: Amplification of the target gene failed.

Summary

We processed a total of 39 samples out of 50 but none of the samples amplified positive. A set of different extraction protocols were implemented to extract high yield DNA from the formalin stored samples. However, isolating high-quality genomic DNA from formalin-fixed PET is difficult because only minimal amounts of intact DNA may be present in the sample (Lin et.al 2002).

Crosslinking is formed between protein and DNA complexes when tissue nucleoproteins is exposed to formalin (Lin et.al 2002).This cross-linking of biomolecules to DNA could directly reduce the PCR amplification efficiency from the genomic DNA of formalin-fixed tissue and therefore significantly decrease the yield of amplifiable genomic DNA (Shapiro et. Al, 2012)

Therefore, we have reported FRD team regarding the issues with formalin stored samples.

Table4. Protocols implemented for extracting DNA from formalin samples

EXTRACTION METHODS	TARGET GENE	AMPLIFICATION
Gene All- Tissue extraction Kit	CO1, 12S	FAILED
ZYMO Research FFPE KIT	12S	FAILED
Qiagen Modified Protocol	12S	FAILED
Extraction using Alkali treatment	12S	FAILED
PHIX 29 (TempliPhi Protocol)	12S	FAILED
Phenol- Chloroform	12S	FAILED

References

- Wu, L., Patten, N., Yamashiro, C. T., & Chui, B. (2002). Extraction and Amplification of DNA from Formalin-Fixed, Paraffin-Embedded Tissues. Applied Immunohistochemistry & Molecular Morphology, 10(3), 269-274. doi:10.1097/00129039-200209000-00015
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- Ivanova, N., Zemlak, T., Hanner, R. And Hebert, P. (2007). Universal primer cocktails for fish DNA barcoding. *Molecular Ecology Notes*, 7(4), pp.544-548.
- Methods in Molecular Biology Walker, J. M. (Ed) 2012