FROM SAMPLE to RAPID DNA and eDNA IDENTIFICATION

An Easy Step-by-Step Guide

Beatriz Rochitti Boza
Ingrid Vasconcellos Bunholi
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Translation: Megan Do
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Beatriz Rochitti Boza
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PREFACE

This book offers a **practical and accessible guide to species identification using DNA or environmental DNA (eDNA)**, covering the process from project design to assay choice, and then from sample collection to genetic analysis. It clearly explains the molecular biology concepts essential to this approach, distinguishes between methods that require sequencing and those that do not, presents the main rapid identification (non-sequencing-based) techniques, and highlights the importance of standardizing procedures to ensure reliable results. Ultimately, **this content aims to assist researchers and environmental professionals in applying these techniques** efficiently and effectively.

The main focus of this book is on cartilaginous fishes (Chondrichthyes); however, many of the concepts and techniques discussed can be applied to other groups as well. Additionally, **this e-book includes four flowcharts that guide readers through each stage of a molecular identification project**, from defining objectives and selecting appropriate techniques to analyzing and validating results.. With this book, we aim to contribute to the dissemination of scientific knowledge and the conservation of marine biodiversity.

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1. WHAT IS MOLECULAR IDENTIFICATION?

Molecular identification is the process of using genetic differences reflected in the DNA to determine the species identity of a sample. As a fundamental tool in conservation biology and related disciplines, it is generally implemented through two main approaches presented below:

1.1 IDENTIFICATION WITH DNA SEQUENCING

This method requires sequencing of DNA extracted from the sample. It provides comprehensive information about genetic variation and is particularly effective for identifying species and detecting rare genetic variants.

1.2 IDENTIFICATION WITHOUT DNA SEQUENCING

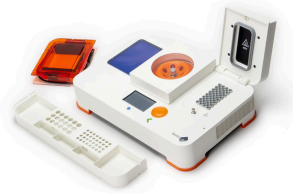
This method does not require laboratory-based sequencing, making it a faster and more cost-effective alternative for molecular identification. These approaches, hereafter referred to as “rapid DNA/eDNA-based ID tools,” enable straightforward DNA detection and, in some cases, quantification.

1. WHAT IS MOLECULAR IDENTIFICATION?

Beyond being faster and more cost-effective, rapid DNA/eDNA-based ID tools are now extending beyond traditional laboratory analysis. Advances in **portable technologies** enable PCR reactions and even sequencing to be carried out directly in the field with compact, user-friendly equipment. In addition to portable PCR platforms, field-deployable sequencing devices such as the MinION (Oxford Nanopore Technologies) allow real-time generation of DNA sequences outside conventional laboratories.

MINIPCR® MINI8

MINIPCR. miniPCR mini8X thermal cycler. Available at: https://www.minipcr.com/product/minipcr-mini8x-thermal-cycler/. Accessed on: May 12, 2025.



BENTO LAB

BENTO BIO. Bento Lab – portable DNA laboratory. Available at: https://bento.bio/product/bento-lab/. Accessed on: May 12, 2025.

BIOMEME FRANKLIN™

BIOMEME. Franklin™ Real-Time PCR Thermocycler. Available at: https://biomeme.com/platforms/franklin/. Accessed on: May 12, 2025.

MINION

OXFORD NANOPORE TECHNOLOGIES. MinION Mk1B – portable nanopore sequencing device. Available at: https://nanoporetech.com/products/sequence/minion-mk1b. Accessed on: Nov 14, 2025.

These **portable devices enable real-time DNA detection**, which is particularly advantageous in scenarios where rapid decisions are essential. These scenarios include, but are not limited to, monitoring threatened species in remote regions, verifying catch identity during fisheries inspections, and supporting wildlife trade enforcement when laboratory access is limited or sample degradation is a concern.

2. CONTEXTUALIZATION:
SHARKS AND RAYS AND THE NEED FOR RAPID IDENTIFICATION TOOLS

Sharks and rays are among the most threatened marine organisms, facing significant pressures due to overfishing and habitat degradation. The illegal capture and trade of threatened species is a serious issue as they are often misidentified and marketed without proper identification, making enforcement and conservation measures even more difficult to implement.

In this context, rapid DNA/eDNA-based ID tools emerge as strategic techniques to enable accurate species detection even of processed products, helping combat illegal fishing and protecting threatened populations. Using rapid techniques, it is possible to obtain quick and inexpensive knowledge of **elasmobranch taxa** that are captured and traded, generating up-to-date data on fisheries activities.

Note: A taxon (plural: taxa) is a biological classification unit that groups organisms with shared characteristics.

2. CONTEXTUALIZATION: SHARKS AND RAYS AND THE NEED FOR RAPID IDENTIFICATION TOOLS

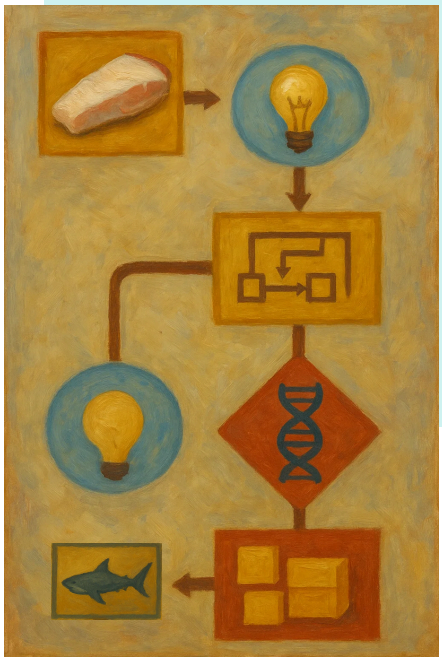
Moreover, applying these rapid techniques to environmental DNA (eDNA) – using environmental samples, such as water, for identification – can facilitate biodiversity monitoring. This is important for developing management strategies that effectively contribute to the conservation and sustainable use of marine resources. Given this, investing in rapid and accessible identification techniques is essential to ensure more efficient and frequent monitoring and enforcement.

Figure 1. Collection and filtration of water samples for environmental DNA (eDNA) analysis using a Smith-Root eDNA Sampler (https://www.smith-root.com/edna/edna-sampler). Image credits: Ingrid Bunholi and Mary Finch.

3. DESIGNING YOUR MOLECULAR ID PROJECT

This section introduces the design stage of a molecular identification project. It provides a practical framework to help researchers, students, and professionals move from an initial problem or idea to a structured project plan.

To provide a clear and practical pathway from concept to implementation, a series of three flowcharts illustrating the molecular ID workflow is presented as follows:



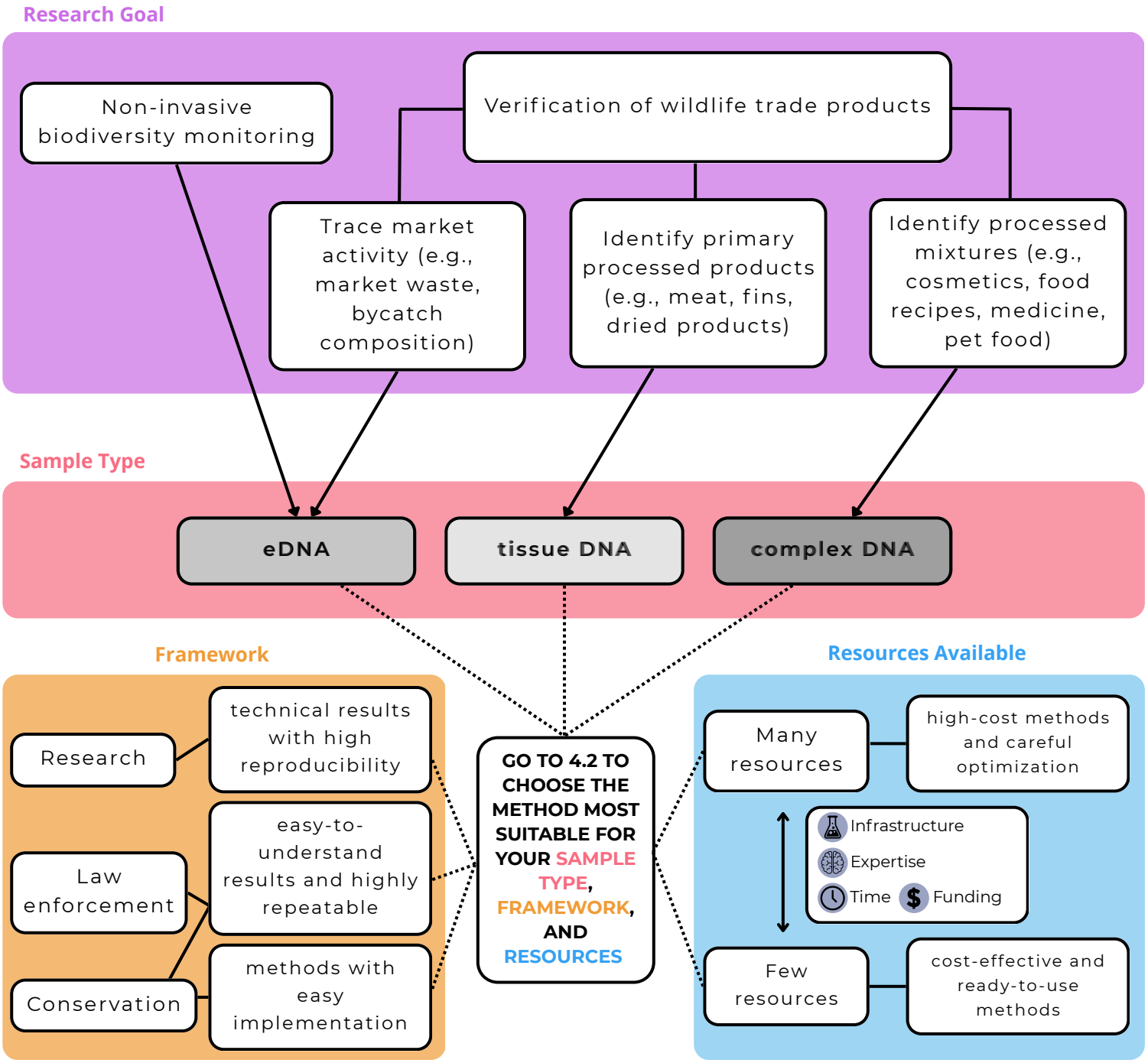
(**i**) how to start a project by approaching an initial idea, question or situation transforming it into a structured action plan, (**ii**) how to select the most appropriate molecular techniques, and (**iii**) how to evaluate and organize available resources.

Finally, in topic 5, “Executing Your Molecular ID Project,” a fourth (**iv**) flowchart is provided to connect the knowledge gained here to the execution of your project, from sample collection to species identification.

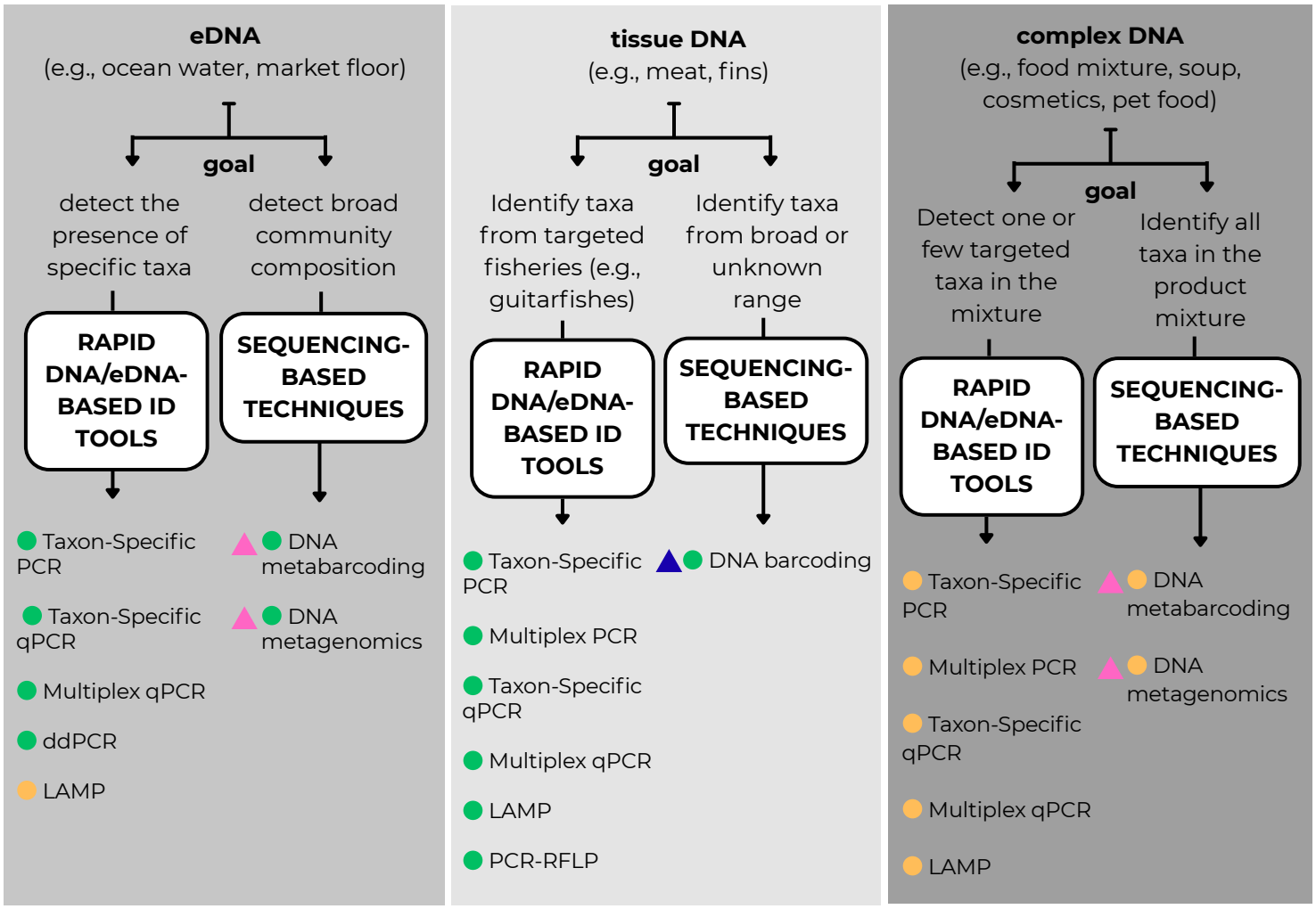
3. DESIGNING YOUR MOLECULAR ID PROJECT

3.1 STARTING A PROJECT

Starting a molecular identification project requires a clear understanding of four fundamental aspects: **what you aim to achieve**, **the type of samples you will use**, **the framework guiding your work**, and **the resources available to you**.



3.2 SELECTING THE RIGHT MOLECULAR TECHNIQUE



Is the setting cost-senstive?
Taxon-Specific PCR, LAMP, PCR-RFLP, Multiplex PCR

Is it easy to development from scratch?
Taxon-Specific PCR, PCR-RFLP

Is quantification needed?
Taxon-Specific qPCR, ddPCR, DNA metagenomics, Multiplex qPCR

Are rapid field results required?
LAMP, Taxon-Specific qPCR, Multiplex qPCR, sequencing with MinION

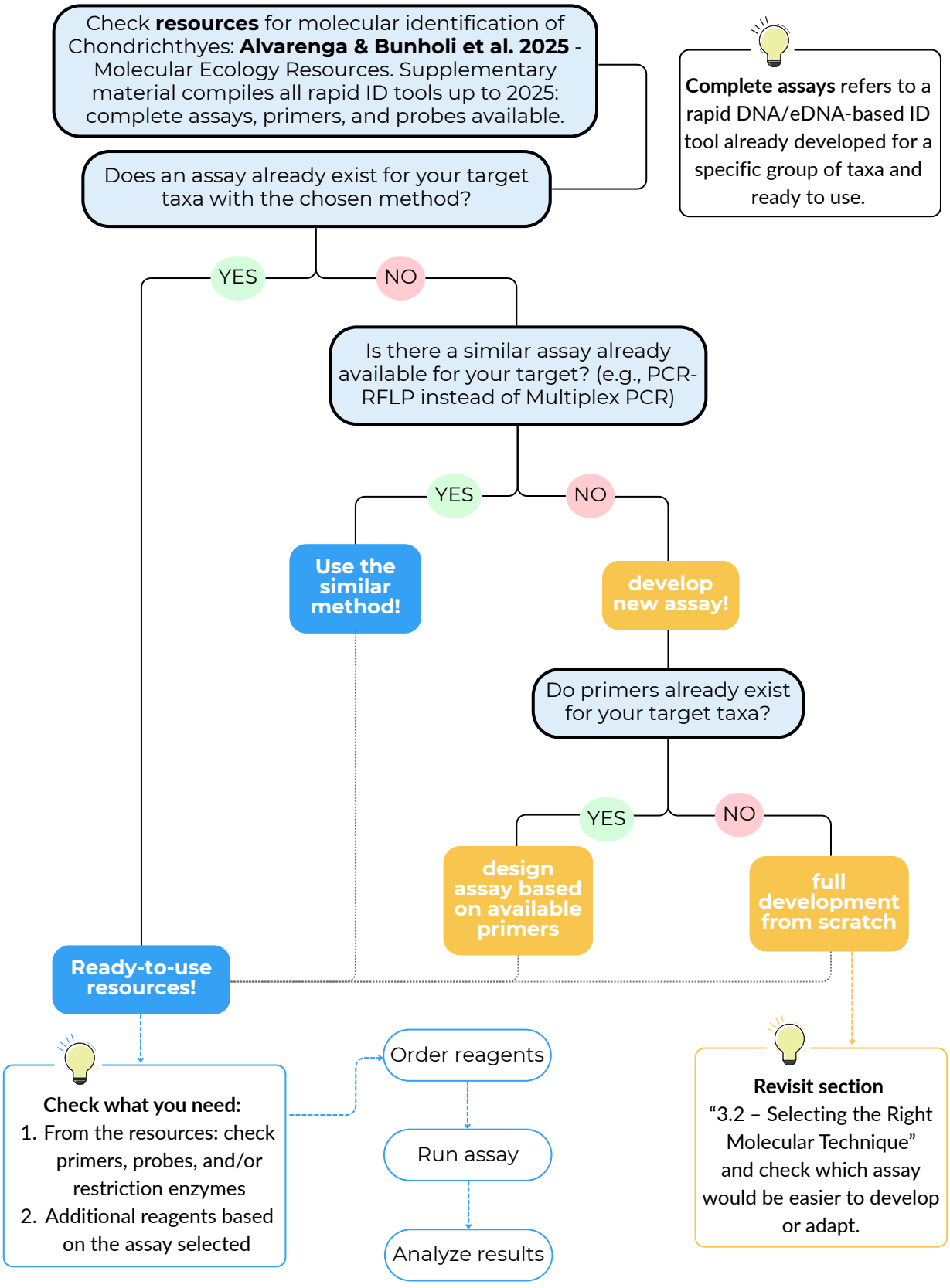
Is sensitivity needed?
ddPCR, Taxon-Specific qPCR

Need to detect a rare species?
ddPCR, Taxon-Specific qPCR

Do all species in the sample need to be identified?
DNA metabarcoding, DNA metagenomics

- Validated for chondrichthyans
- Not validated for chondrichthyans
- NGS sequencing
- Sanger sequencing

3.3 WHAT TOOLS ARE AVAILABLE?



4. MOLECULAR ID BASICS: WHAT YOU SHOULD KNOW TO DESIGN YOUR PROJECT

After outlining how to design a molecular identification project, the next step is to understand the **essential molecular concepts** that will guide your decisions. A solid understanding of basic molecular biology is key to selecting the most appropriate methods and ensuring a robust project design.

This section provides a simplified overview of fundamental concepts and highlights some of the most widely used and emerging rapid DNA/eDNA-based identification tools. Together, these insights provide the foundation needed to align your project plan with the molecular approaches that make it possible.

4.1 WHAT IS PCR (POLYMERASE CHAIN REACTION)?

- **Simple Definition**

PCR is a technique used to make multiple copies of a specific region of DNA. We could say that it works similarly to a photocopier.

These copies may have specific sizes, determined by primers (see definition below), to help identify species or specific groups, serving as the basis for many molecular identification techniques.

4. MOLECULAR ID BASICS: WHAT YOU SHOULD KNOW TO DESIGN YOUR PROJECT

4.1 WHAT IS PCR (POLYMERASE CHAIN REACTION)?

- **Main Reagents**

Template DNA - DNA strand that contains the sequence of interest to be amplified/copied.

Primers - Short, single-stranded sequences that bind to complementary regions of the template DNA, identifying the region to be amplified.

Polymerase - An enzyme that synthesizes new DNA strands by adding complementary nucleotides to the template strand, starting at the primer region. *Taq polymerase* is the enzyme of choice for PCR because it can 'survive' the heat needed to unzip the DNA, something most enzymes cannot do.

Nucleotides (deoxyribonucleoside triphosphates: **dNTPs**) - The basic building blocks of DNA. Each nucleotide consists of three components: a nitrogenous base (adenine = **A**, thymine = **T**, cytosine = **C**, or guanine = **G**), a five-carbon sugar (deoxyribose), and a phosphate group.

Magnesium chloride (**$MgCl_2$**) - Provides magnesium ions that act as cofactors for DNA polymerase, influencing enzyme activity, primer binding, and amplification specificity.

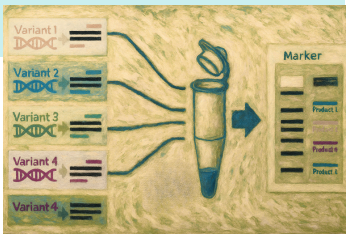
Buffer - A stabilizing solution that helps maintain the pH and optimal conditions necessary for the polymerase enzyme to work properly, ensuring the reaction occurs efficiently.

4. MOLECULAR ID BASICS: WHAT YOU SHOULD KNOW TO DESIGN YOUR PROJECT

4.2 PCR VARIATIONS

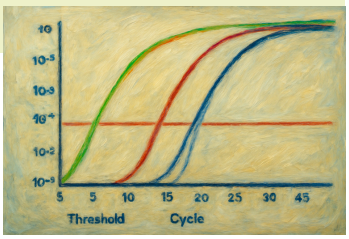
- **Multiplex PCR**

Test the presence of multiple regions of DNA at the same time by using multiple primers.



- **Real-time PCR (qPCR)**

Allows for real-time quantification of DNA using specific primers. Probes can be added to increase specificity, especially for eDNA samples, but their use is not strictly limited to eDNA; both tissue and eDNA samples can be analyzed with primers alone or combined with probes.



- **Droplet-Digital PCR (ddPCR)**

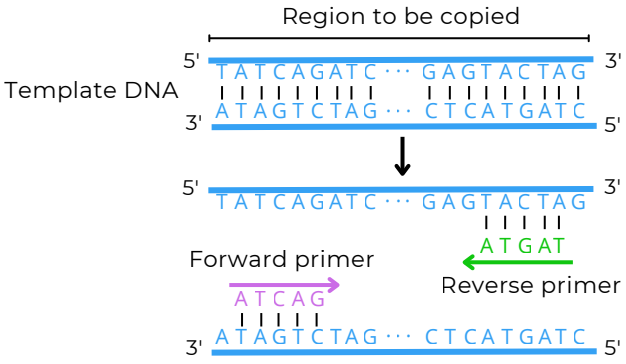
Amplifies DNA through thousands of microscopic droplets, ensuring precise quantification of eDNA in low quantities. It requires specific primers and probes.

4.3 PRIMER VS. PROBE: WHAT IS THE DIFFERENCE?

Primers and probes are used in molecular biology techniques, primarily for PCR and its variants, with probes more commonly used for qPCR and ddPCR.

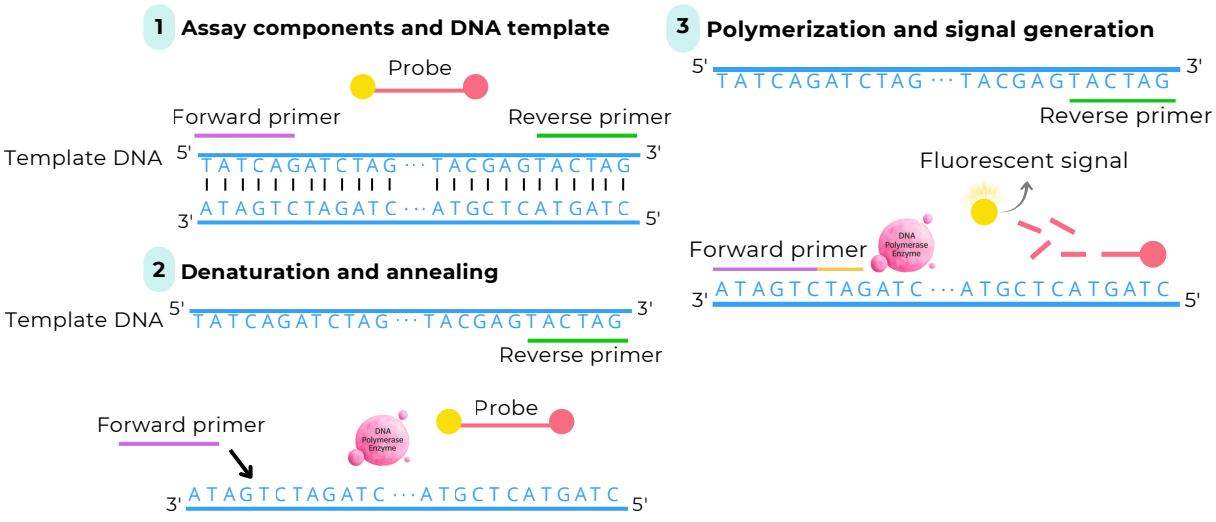
- **Primers**

These are short DNA sequences that serve as starting points for DNA amplification in PCR. They bind to the target sequence and tell the enzyme (polymerase) where to start copying the DNA.



- **Probes**

These are short DNA sequences that specifically bind to the target DNA and contain a fluorescent marker. They are used to increase the specificity of eDNA sample identification and quantification in both qPCR and ddPCR applications.



4. MOLECULAR ID BASICS: WHAT YOU SHOULD KNOW TO DESIGN YOUR PROJECT

4.4 SIMPLIFIED GENETICS – UNDERSTANDING KEY CONCEPTS

- **Bioinformatics**

Bioinformatics is a scientific field that combines biology and computing to analyze large biological datasets, such as DNA, RNA, and protein sequences. It is essential for streamlining genetic analyses and assists with:

- Gene and mutation identification;
- Sequence comparison between different organisms;
- Primer design for PCR techniques;
- *In silico* experiments and simulations;
- Statistics for analyzing genetic variation and sequencing data.

- **Cells**

A cell is the smallest functional unit of living organisms. It acts like a small factory, carrying out essential activities such as energy production, growth, and reproduction. Inside the cells, there is a very important structure called the nucleus (in eukaryotic cells) where the DNA is housed, storing and transmitting genetic information across generations.

It is important to remember that there are **two main types of cells**:

Prokaryotic cells 

Simpler cells without a defined nucleus where genetic material (DNA or RNA) is free-floating inside the cell; prokaryotes include Bacteria and Archaea.

Eukaryotic cells 

More complex cells with a membrane-bound nucleus and specialized organelles; eukaryotes include animals, plants, fungi, and protists.

4. MOLECULAR ID BASICS: WHAT YOU SHOULD KNOW TO DESIGN YOUR PROJECT

4.4 SIMPLIFIED GENETICS – UNDERSTANDING KEY CONCEPTS

- **Chain reaction**

A chain reaction is a process where an initial action triggers a sequence of successive events. A well-known example is PCR (Polymerase Chain Reaction); as discussed earlier in this book, PCR copies a DNA fragment millions of times.

- **Contamination**

Contamination in molecular analyses can arise from several sources and compromise the accuracy of results. It occurs when unwanted substances or materials interfere with the sample or analysis process, leading to errors or false positives/negatives. Examples of contamination include:

- Environmental contamination (e.g., DNA/RNA residues from previous samples or aerosols in the workspace);
- Contamination from reagents or equipment (e.g., expired reagents or improperly maintained equipment);
- Pipetting and handling errors (e.g., improperly calibrated pipettes, poorly fitted pipette tips, or handling samples without gloves).

- **DNA (Deoxyribonucleic Acid)**

DNA is a biological macromolecule that acts as the primary genetic material of all living organisms and some viruses. DNA is a polymer composed of two long chains (strands) formed from units called nucleotides, which coil around themselves to form an antiparallel double helix structure.

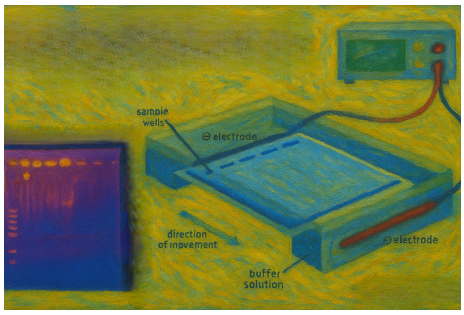
4. MOLECULAR ID BASICS: WHAT YOU SHOULD KNOW TO DESIGN YOUR PROJECT

4.4 SIMPLIFIED GENETICS – UNDERSTANDING KEY CONCEPTS

- **eDNA (Environmental DNA)**

eDNA is genetic material shed by organisms into the environment, such as in water, soil, or air. It can be analyzed to detect the presence of a species in an ecosystem without the need to capture or take samples from them directly.

- **Electrophoresis**



Electrophoresis is a technique used to separate DNA, RNA, or proteins based on their charge and size. It is commonly performed in an agarose gel with saline buffers such as TAE (Tris-acetate-EDTA) or TBE (Tris-borate-EDTA), which help maintain a stable pH during the run. Nucleic acids have a negative charge due to their phosphate backbone and migrate toward the positive electrode (anode) when an electric current is applied. Smaller molecules move more easily and faster through the gel pores, while larger molecules move more slowly, allowing visualization and comparison according to molecular size.

- **Enzyme**

Enzymes are proteins that act as “machines,” performing essential chemical reactions such as DNA replication or cleavage. Restriction enzymes, for example, recognize specific nucleotide sequences in DNA and cut at those exact sites, generating fragments useful for molecular identification. DNA polymerase, on the other hand, is a enzyme that synthesizes new DNA by reading an existing template strand and adding complementary nucleotides.

4. MOLECULAR ID BASICS: WHAT YOU SHOULD KNOW TO DESIGN YOUR PROJECT

4.4 SIMPLIFIED GENETICS – UNDERSTANDING KEY CONCEPTS

- **Genetic amplification**

Genetic amplification is the process of creating multiple copies of a specific DNA fragment. It is useful for gene studies, organism identification, and even forensic DNA testing.

- **Molecular marker**

A molecular marker is a specific DNA sequence used to identify genetic traits of an organism, functioning like a “biological barcode.” Molecular markers are widely used to differentiate species, study relationships, or diagnose diseases.

- **PCR inhibitor**

PCR inhibitors are substances, such as humic acid or dissolved metals, found in environmental samples that can interfere with polymerase and primer function, reducing PCR efficiency and causing problems in real-time methods like qPCR.

- **RNA (Ribonucleic Acid)**

RNA is a single-stranded molecule that copies information from DNA and assists in protein production, a process that is crucial for an organism’s functioning.

- **Sequencing**

Sequencing is the process of determining the exact order of nucleotides (**A**, **T**/**U**, **C**, and **G**) in a DNA or RNA molecule. In molecular identification and eDNA studies, sequencing allows scientists to compare genetic sequences with reference databases to identify species, detect genetic variation, and assess biodiversity.

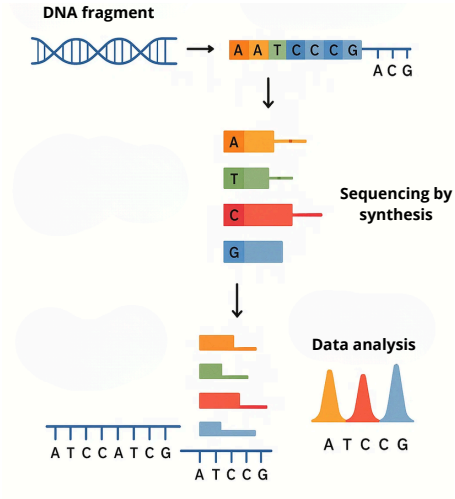
4. MOLECULAR ID BASICS: WHAT YOU SHOULD KNOW TO DESIGN YOUR PROJECT

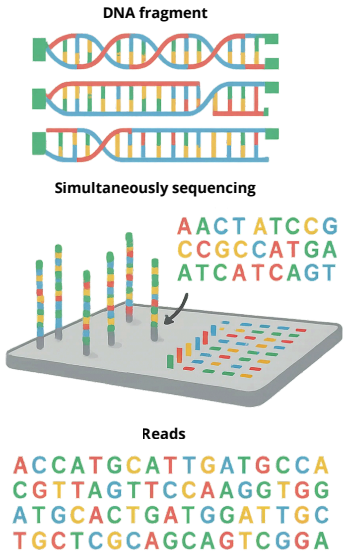
4.4 SIMPLIFIED GENETICS – UNDERSTANDING KEY CONCEPTS

Several sequencing methods are available, from classic **Sanger sequencing**, which reads one DNA fragment at a time, to **High-Throughput Sequencing** (**HTS**), also known as **Next Generation Sequencing** (**NGS**), which is capable of processing millions of fragments simultaneously.

Sanger sequencing:

A classic method that determines the nucleotide order of a DNA fragment by synthesizing new strands using modified nucleotides that stop the reaction at specific bases. This approach is highly accurate but limited in throughput as it sequences one DNA fragment at a time.





High-Throughput Sequencing (HTS):

A set of high-throughput methods that allow for the sequencing of millions of DNA or RNA fragments simultaneously. Here, the fragments are amplified and read base by base, usually using fluorescent signals emitted during nucleotide incorporation. HTS enables large-scale and rapid sequencing that is suitable for a variety of genomic applications.

While Sanger sequencing is simpler and cost-effective for small, straightforward identification projects, HTS provides greater depth and resolution for complex analyses such as metabarcoding and tracking the population origin of a sample.

4.5 RAPID DNA/eDNA-BASED ID TOOLS

TAXON-SPECIFIC PCR

Definition:

A PCR technique designed to detect specific taxa using primers that target DNA regions unique to a particular taxonomic group.

Target samples:

Tissue, eDNA, and complex DNA.

Practical applications:

Identifying which species a tissue sample belongs to or checking if a specific species is present in an environmental or food sample.

How to interpret the results:

If the target DNA is present, a visible amplification band at the expected fragment size will appear on the electrophoresis gel.

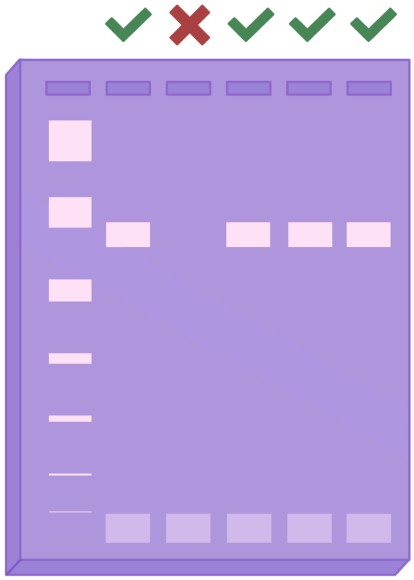


Figure 2. Schematic illustration of a taxon-specific PCR agarose gel containing five samples. The first lane shows the molecular size standard (ladder). Lanes 2–6 display PCR amplification products, with sample 2 showing a failed amplification, indicating absence of the target DNA. Created with BioRender.com.

Limitations: Highly sensitive to primer design errors and may produce false negatives or detect similar species if primers are poorly designed. Low sensitivity to small amounts of target DNA, limiting its use in eDNA samples (e.g., amplification may not be visible on agarose gels).

Recommendations: Validate primers before use and always include positive and negative controls.

4.5 RAPID DNA/eDNA-BASED ID TOOLS

MULTIPLEX-PCR

Definition:

A PCR technique that can amplify multiple DNA regions simultaneously by using several primers in a single reaction.

Target samples:

Tissue and complex DNA.

Practical applications:

Enables rapid detection — ideal for regular trade checks and monitoring programs.

How to interpret the results:

Each species produces a specific band pattern that can be observed on an agarose gel.

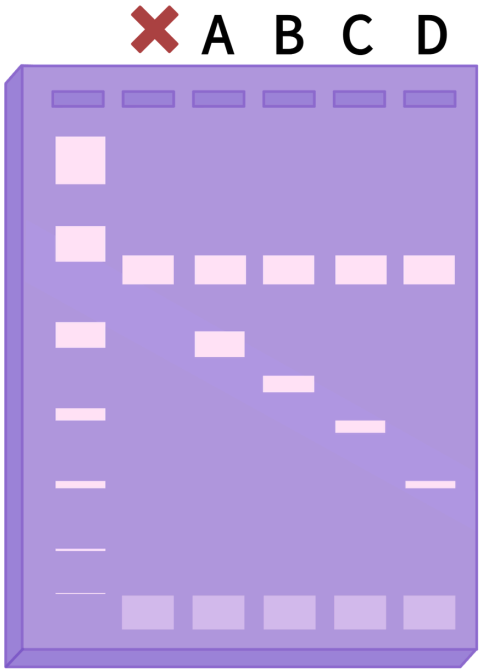


Figure 3. Schematic illustration of a multiplex PCR agarose gel containing five samples. The first lane shows the molecular size standard (ladder). Lanes 2–6 display a universal band and amplification products of different sizes, corresponding to multiple target regions amplified simultaneously in each reaction. Each band pattern represents a different species (A, B, C, and D). Lane 1 illustrates a failed identification, in which only the universal band was amplified. Created with BioRender.com.

Limitations: Risk of non-specific amplification; closely related targets or mixed samples need careful primer design and optimization.

Recommendations: Use validated primers from established databases, check literature before developing new assays, and defer to multiplex qPCR for higher specificity and PCR–RFLP (see next page) for closely related taxa.

4. MOLECULAR ID BASICS: WHAT YOU SHOULD KNOW TO DESIGN YOUR PROJECT

4.5 RAPID DNA/eDNA-BASED ID TOOLS

PCR-RFLP

Definition:

A technique that uses restriction enzymes to cut the amplified DNA at specific recognition sites, generating fragment patterns that enable species identification.

Target samples:

Tissue.

Practical applications:

Differentiating closely related taxa or species with limited genetic divergence since differences at restriction sites can provide specific markers that allow species identification, even in otherwise conserved regions.

How to interpret the results:

The digested DNA generates a fragment pattern that can be analyzed on an electrophoresis gel to determine the species.

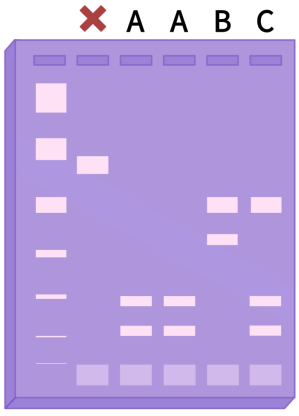


Figure 4. Schematic illustration of a PCR–RFLP agarose gel containing five samples. The first lane shows the molecular size standard (ladder). Lanes 2–6 exhibit distinct restriction fragment patterns, reflecting differences in the presence or absence of specific restriction sites among samples. Three different species (A, B, and C) are represented, each showing a characteristic banding pattern. Column 1 represents a failed amplification. Created with BioRender.com.

Limitations: Requires prior knowledge of restriction sites, which may limit use across taxa.

Recommendations: Select and test restriction enzymes carefully and compare digestion patterns with reference databases for accurate identification.

4. MOLECULAR ID BASICS: WHAT YOU SHOULD KNOW TO DESIGN YOUR PROJECT

4.5 RAPID DNA/eDNA-BASED ID TOOLS

TAXON-SPECIFC qPCR

Definition:

A technique that amplifies and quantifies DNA/eDNA simultaneously, using fluorescence to detect the target species' presence in real time.

Target samples:

Tissue, eDNA, and complex DNA.

Practical applications:

Species identification and biodiversity monitoring. Portable machines enable on-site identification (see page 2 for more details).

How to interpret the results:

The earlier the cycle number at which the amplification curve crosses the detection threshold, the higher the initial amount of target DNA (or eDNA). The signal comes from dyes or probes that emit light during amplification, enabling real-time monitoring.

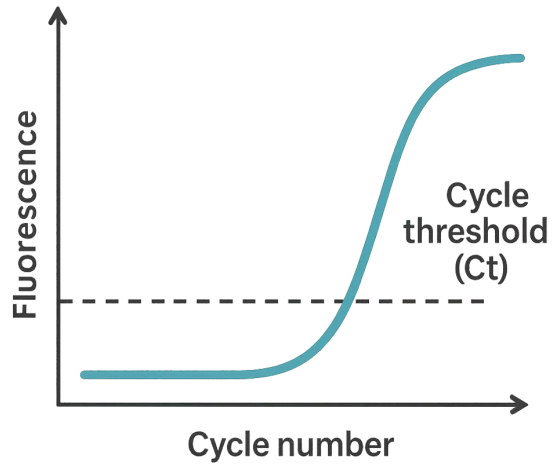


Figure 5. Schematic illustration of a standard qPCR amplification plot. The horizontal line represents the fluorescence threshold, and the vertical line (Ct) indicates the cycle number at which the fluorescence of the target DNA crosses this threshold and becomes detectable. Created with Sora.

Limitations: Less sensitive to rare taxa in eDNA samples compared to ddPCR (see below); susceptible to PCR inhibition.

Recommendations: Employ strict controls and specific primers and probes. Validate primers and probes *in silico* using computer software, such as PrimerBlast, and *in vitro* with tissue samples of the target species and co-occuring species before analyses.

4.5 RAPID DNA/eDNA-BASED ID TOOLS

MULTIPLEX qPCR

Definition:

A version of qPCR that amplifies multiple DNA sequences simultaneously using multiple primer sets. Fluorescent dyes or labeled probes allow the detection of each target in a single reaction.

Target samples:

Tissue and eDNA.

Practical applications:

Detecting and quantifying multiple taxa from tissue and eDNA samples.

How to interpret the results:

Each target species generates a distinct amplification curve, allowing for the simultaneous detection of multiple species in complex samples. Cycle threshold (Ct) values indicate presence of the target DNA.

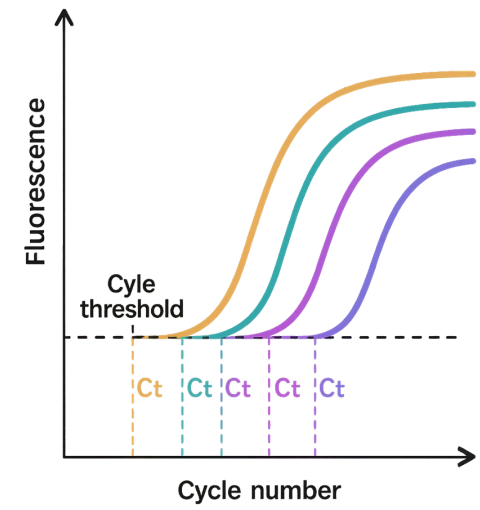


Figure 6. Schematic illustration of a multiplex qPCR amplification plot showing multiple amplification curves of different colors, each representing a distinct target DNA sequence (i.e., different species). The horizontal black line represents the fluorescence threshold, and the vertical lines (Ct) indicate the cycle number at which each target DNA crosses this threshold and becomes detectable. Created with Sora.

Limitations: Complex assay design, higher risk of non-specific amplification, and costly compared to multiplex PCR.

Recommendations: Use validated primers and probes, optimize reactions, and include all controls. For simpler samples, consider cheaper options like multiplex PCR or PCR–RFLP.

4.5 RAPID DNA/eDNA-BASED ID TOOLS

DROPLET-DIGITAL (ddPCR)

Definition:

A highly sensitive DNA quantification method in which the PCR reaction is partitioned into thousands of oil-encapsulated nanodroplets, each acting as an independent reaction.

Target samples:

eDNA.

Practical applications:

Biodiversity monitoring, especially for rare or low-abundance species

How to interpret the results:

Positive droplets = target DNA detected. Negative droplets = target DNA not detected (absence or amplification failure). A higher number of positive droplets indicates a higher concentration of target DNA.

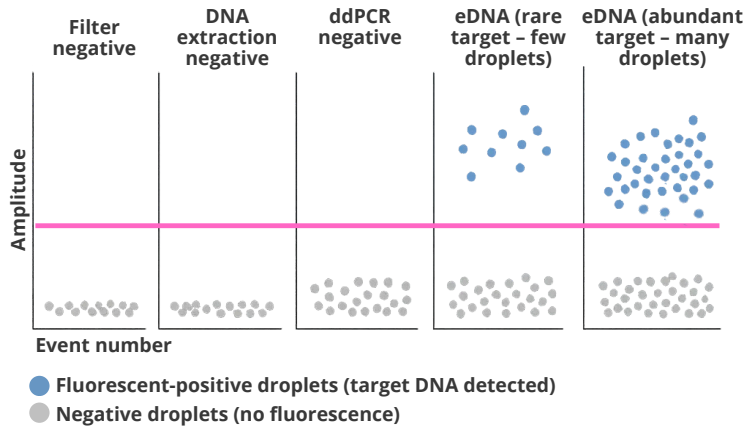


Figure 7. Schematic illustration of ddPCR fluorescence amplitude plots (fluorescence amplitude vs. event number) for five sample types: filter negative (no sample filtered, control for field contamination), DNA extraction negative (no DNA template, control for extraction contamination), ddPCR negative (no DNA template, control for PCR contamination), eDNA – rare (few positive droplets), and eDNA – abundant (many positive droplets). The pink line indicates the assay detection threshold. Created with Sora and Canva.

Limitations: Costly and requires specialized equipment and training.

Recommendations: Use ddPCR for low-abundance or inhibitor-rich samples; validate primers and probes both *in silico* and *in vitro* with target and co-occurring species before analyses.

4.5 RAPID DNA/eDNA-BASED ID TOOLS

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

Definition:

A fast technique that amplifies DNA at a constant temperature without the need for PCR amplification or a thermocycler.

Target samples:

Tissue, eDNA.

Practical applications:

Ideal for rapid taxa identification, especially for fieldwork or onsite identification.

How to interpret the results:

A color change or precipitate formation indicates the presence of the target species' DNA.

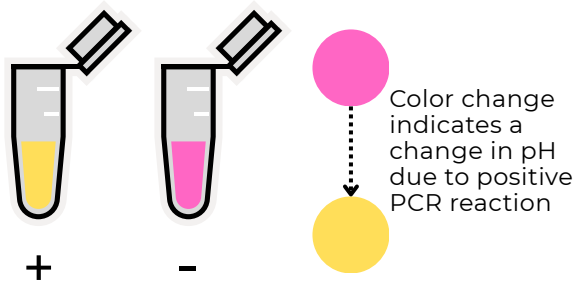


Figure 8. Schematic illustration of positive LAMP reaction with the color change from pink to yellow, indicating a change in pH and the presence of target species DNA in species-specific assays. Created with Canva.

Limitations: Sensitive to contamination and may be less specific than traditional PCR techniques.

Recommendations: Use well-designed primers and validate assays before large-scale application.

5. EXECUTING YOUR MOLECULAR ID PROJECT

1. SAMPLE COLLECTION

Tissue (e.g., muscle, fin)

- Use clean instruments and cut ~0.5 cm² of tissue
- Preserve in ≥ 92% ethanol and refrigerate sample

Note: The same principles can be applied to complex samples (e.g., food mixture, soup, cosmetics, pet food)

eDNA (e.g., water filter, sediment)

- Collect ~1–5 L of water or ~5 g of sediment using sterilized instruments
- Preserve sample with RNAlater (or a similar reagent) or freeze immediately

2. RECORDING AND LABELING

- Record the date, location, coordinates, source, and environmental conditions (for eDNA) or sex and body size (for tissue)
- Photograph tissue samples and ensure all records are complete and clear

3. DNA / eDNA EXTRACTION

Requires centrifuge, micropipettes, and specific extraction reagents.

- Use commercial kits or homemade protocols
- Remove inhibitors if working with environmental samples

4. ANALYZING DNA / eDNA

- Choose a suitable molecular technique for analyses based on section "*4.2 Selecting the Right Molecular Technique*"
- Follow the specific protocol, using the correct reagents, primers, and/or probes
- Ensure all controls are included to check for contamination and assay performance

Revisit section "4.5 – Rapid DNA/eDNA-based ID Tools."

5. RESULTS ANALYSIS

- Interpretation depends on the technique used, which may involve amplification signals, sequence comparisons, or marker-specific results

6. SPECIES CONFIRMATION

- Evaluate your results to confirm the presence or absence of the target species
- If results are ambiguous, repeat the assay, apply an alternative molecular technique, or compare obtained sequences with reference databases
- Record all confirmed results to complete the molecular ID workflow

5. EXECUTING YOUR MOLECULAR ID PROJECT

5.1 HOW TO COLLECT AND PRESERVE SAMPLES FOR IDENTIFICATION

- **Tissue**

(Muscle, liver, or fin tissue)

Storage: One tissue sample per container to avoid contamination.

Recommendations:

Tissue size: Approximately 0.5 cm^2;

Use clean cutting instruments;

Clean materials for sample collection and handling (e.g., scissors, tweezers, etc.) with a 70% or higher ethanol solution between samples.

Tissue to fixative ratio: It is crucial to ensure that the tissue is completely immersed in ethanol, which will act as the fixative to preserve the sample. Whenever possible, it is recommended that the container holding the sample be kept refrigerated to assist in preservation and DNA quality.

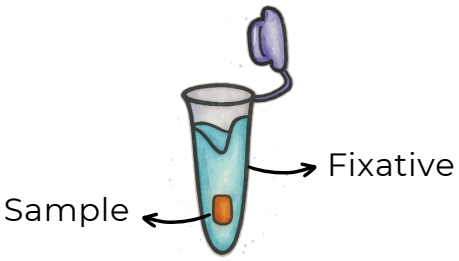
The sample should be fixed in ethanol ≥92%, as lower concentrations compromise preservation. Tissue volume should occupy no more than ¼ of the container, allowing the ethanol to fully surround the sample.

5. EXECUTING YOUR MOLECULAR ID PROJECT

5.1 HOW TO COLLECT AND PRESERVE SAMPLES FOR IDENTIFICATION

A 1.5 mL microtube is sufficient for accommodating roughly 0.5 cm^2 of tissue. Ensure the sample moves freely in ethanol when the tube is gently agitated, avoiding compression against the bottom or sides of the microtube or entrapment under the cap.

Example:



It is recommended to change the ethanol fixative at least three times after immersing the sample in the fixative, especially if the sample is not being processed immediately. This is because water can be lost from the sample into the solution, lowering the alcohol concentration of the fixative.

- **Environmental DNA (eDNA)**

(Water filter or sediment)

Sample size: Approximately 1-5 L of water or 5 g of sediment.

Recommendations:

Sterilize instruments and materials with 10% bleach before use;

Filters or sediment should be immediately frozen or preserved with RNAlater (or a similar reagent) upon collection.

see Malmstrom (2015) for a suggested preparation: https://doi.org/10.17504/protocols.io.c56y9d

Add a negative control (e.g., Milli-Q water or distilled water) at each collection site for contamination control;

Include field replicates when possible to increase the probability of species detection.

5. EXECUTING YOUR MOLECULAR ID PROJECT

5.2 HOW TO PROPERLY RECORD SAMPLES COLLECTED

- **Correct record-keeping**

For **tissue samples**, labels should be placed directly on the tube using permanent markers, and notes can also be written in pencil on tracing paper or another liquid-resistant paper stored with the sample. For **eDNA samples**, the same labeling approach applies; ensure all information is recorded before freezing or adding preservatives to avoid label damage.

Note 1: Since the fixative and preservative used for tissue samples is ethanol, notes on paper or microtubes made with pens, permanent markers, or other types of markers often smudge or fade, making sample identification unfeasible.

Note 2: Samples with illegible or smudged codes, or without identification, almost always lose their usefulness and must be discarded, as it becomes nearly impossible to trace which organism or site they were collected from.

Each sample should have a numerical or alphanumeric code on the microtube.
Record metadata in a notebook or digital file, including:
Date and location of collection (city, state, and site name – e.g., market, river, lake, etc.);
Geographic coordinates (if available);
For **tissue samples**: origin/source (e.g., fishing, market) and specimen sex (if determinable);
For **eDNA samples** (water or sediment): environmental conditions (e.g., temperature, depth, substrate type), volume of water filtered or sediment collected, and sampling depth.

5.3 PHOTOGRAPHIC RECORD

Whenever possible, photographs of the specimen, or parts of the specimen (e.g., fins), should be taken to create a visual record and aid in species identification, especially when collecting tissue samples for laboratory analysis.

- **Required Equipment**

High-resolution camera or smartphone;

Auxiliary lighting source for dark environments (e.g., flashlight or external flash, if necessary);

A reference scale object (a ruler or tape measure that contrasts with the background color is ideal, but in the absence of these, a coin, pen cap, pen, pencil, or any other object that can be used to estimate size is sufficient).

- **Steps for photography**

Place the specimen on a clean, contrasting background (e.g., a white or black surface), with the scale placed next to the specimen for size reference (ensure the scale is framed within the photograph);

Example:

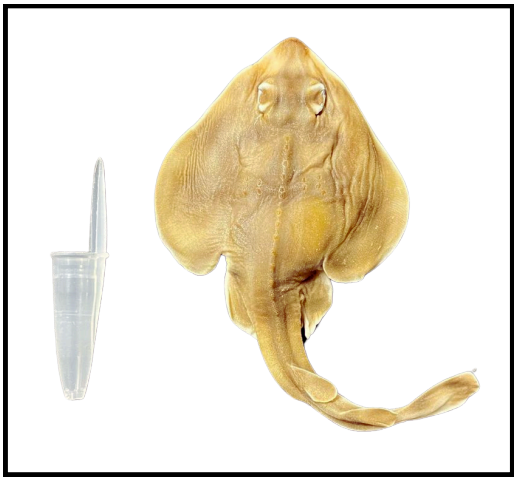


Figure 9. Embryo of *Zapteryx brevirostris* in dorsal view. The pen cap beside the specimen serves as a size reference. Image credits: Beatriz Rochitti Boza.

5. EXECUTING YOUR MOLECULAR ID PROJECT

5.3 PHOTOGRAPHIC RECORD

Name image files in a standardized way (e.g., “Sample Code_Species_Location_Collector_Date”);

Photograph each specimen so images can be linked to the corresponding sample (e.g., include a tag or label photos sequentially, such as PP01a, PP01b);

Capture the entire specimen from lateral, dorsal, and ventral views, highlighting key features such as the head/rostrum region (i.e., mouth, eyes, nostrils, teeth), pelvic region, fins, and distinctive traits (e.g., spines, tubercles, or caudal notch);

For rays, try to capture images of the dorsal, ventral, head/rostrum region (i.e., mouth, eyes, nostrils, spiracle), and pelvic region with fins.

Example:

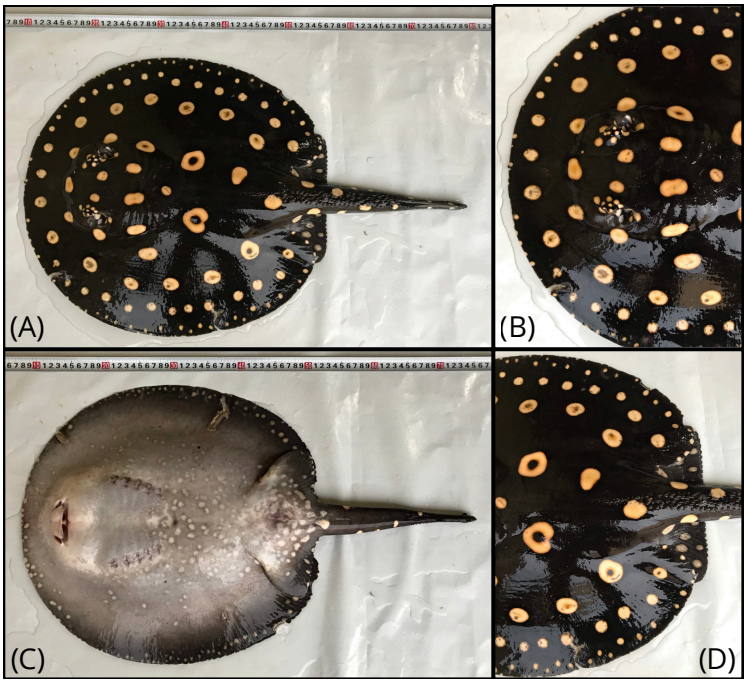
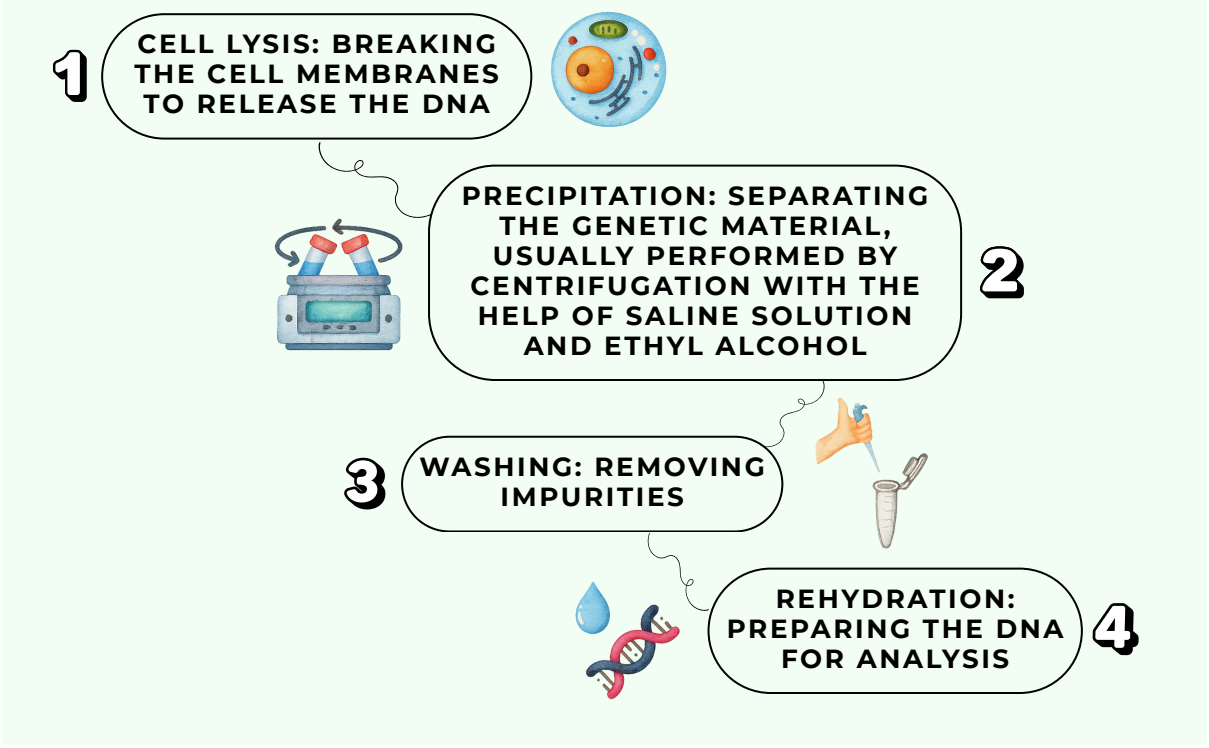


Figure 10. *Potamotrygon leopoldi* - (A) dorsal general view, (B) anterior dorsal region, (C) ventral general view, and (D) posterior dorsal region. The ruler in these images serves as a size reference for the specimen. Image credits: Patricia Charvet.

Remember: A good photographic record will greatly assist in identifying the specimen or its parts.

5.4 HOW TO ACCESS THE SAMPLE'S DNA



The use of commercial kits can optimize DNA washing, especially for eDNA. These kits typically use columns with specific membranes that retain the DNA while unwanted molecules are eliminated, making the process more efficient and reproducible.

Homemade DNA extraction protocols can also be used, greatly reducing costs. For example, an optimized CTAB-based protocol for different tissue types is available (Godinez-Vidal & Groen, 2025).

eDNA samples can contain many inhibitors (e.g., humic acid, sediments, dissolved metals, etc.) that can hinder the quality of the extracted DNA. Therefore, extraction protocols that eliminate these inhibitors ensure better quality in the analyses (amplification).

Godinez-Vidal, R., & Groen, S. (2025). Optimized CTAB DNA extraction for different tissues (Version 1). Department of Nematology, University of California Riverside. Protocols.io.

5. EXECUTING YOUR MOLECULAR ID PROJECT

Before you start analyzing your DNA sample, make sure you have everything you need. See the **checklist** below:

Quick Checklist

- ☑ **DNA sample** ready and properly stored
- ☑ **Technique chosen** (e.g., PCR, qPCR, ddPCR, LAMP, multiplex PCR, PCR-RFLP, multiplex qPCR)
- ☑ **Reagents prepared** (e.g., primers/probes, enzymes, buffers, consumables)
- ☑ **Equipment available** (e.g., thermocycler, qPCR/ddPCR machine, electrophoresis, if needed)
- ☑ **Controls set up** (positive & negative)
- ☑ **Sample info recorded** (e.g., location, date, preservation method, species)
- ☑ **Time & budget planned**
- ☑ **Lab support confirmed** (if access to infrastructure is limited)

For step-by-step details on each technique, revisit Section **4.5 – Rapid DNA/eDNA-based ID Tools.**

5. EXECUTING YOUR MOLECULAR ID PROJECT

5.5 HOW TO ANALYZE YOUR DNA SAMPLE

After completing the checklist and ensuring that you have all of the necessary materials, if you do not have access to the required equipment, consider where to analyze your DNA sample. The best approach is to contact a specialized lab or research group. Universities, research centers, museums, and scientific collections often provide molecular ID services or collaborate with external researchers. Searching recent publications or platforms like ResearchGate and Google Scholar can help identify active groups for your target species.

Before reaching out, gather all relevant information about your sample and collection details (location, date, method, preservation), suspected species, and purpose of analysis (research, monitoring, conservation, or law enforcement). Also, ensure access to necessary reagents (primers, probes, enzymes, buffers) through purchase or collaboration. Some institutions may only accept samples through partnerships or charge a fee, so always confirm availability and procedures in advance.

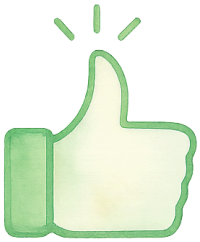
5.6 HOW TO INTERPRET AND VALIDATE YOUR RESULTS

Once the sample DNA has been analyzed, the next step is to interpret the results. How you do this depends on the chosen molecular technique. Each method has its own way of indicating the presence, absence, or quantity of your target species.

Revisit Section **4.5 – Rapid DNA/eDNA-based ID Tools** for guidance on interpreting results specific to each technique. Doing so will help ensure you understand what your data mean and how to apply it correctly in research, monitoring, or conservation contexts.

After interpreting your results, it is essential to validate the identification to ensure accuracy. This process may include adding replicates or sequencing a subset of samples to confirm that the rapid detection results are consistent.

Validation helps reduce errors and increases confidence in your identifications. By completing these steps, you finalize the molecular ID workflow and achieve species confirmation, documenting the presence or absence of your target species as indicated in the flowchart.



6. FURTHER READING

BELOW ARE SOME SUGGESTED TEXTS FOR ADDITIONAL INFORMATION ABOUT DIFFERENT TAXONOMIC GROUPS AND MOLECULAR TECHNIQUES.

This e-book was primarily inspired by the recent paper:

Alvarenga & Bunholi et al. (2025). Rapid DNA/eDNA-Based ID Tools for Improved Chondrichthyan Monitoring and Management. Molecular Ecology Resources, e70044. https://doi.org/10.1111/1755-0998.70044

The work provides a comprehensive **overview of rapid DNA and eDNA-based identification tools**, highlighting their essential role in monitoring biodiversity and regulating the trade of species of conservation concern, particularly Chondrichthyes. It compiles current methods, available taxon-specific primers, and identifies key gaps in coverage, offering practical guidance for effective species identification.

As such, this paper serves as a **starting point** for readers interested in the **practical application of molecular tools in elasmobranch conservation**. By exploring its insights, readers can understand the methods, challenges, and opportunities in applying rapid DNA/eDNA approaches to real-world monitoring and management of sharks and rays. **This paper is the first recommended reading in this section**.

6. FURTHER READING

BELOW ARE SOME SUGGESTED TEXTS FOR ADDITIONAL INFORMATION ABOUT DIFFERENT TAXONOMIC GROUPS AND MOLECULAR TECHNIQUES.

Cartilaginous Fishes

Alvarenga et al. (2024). Fifteen years of elasmobranchs trade unveiled by DNA tools: Lessons for enhanced monitoring and conservation actions. *Biological Conservation*, 292, 110543. https://doi.org/10.1016/j.biocon.2024.110543

Prasetyo et al. (2024). SHARKlock holmes: Applications of DNA forensic in tackling illegal trade of sharks and rays in Southeast Asia Region. *BIO Web of Conferences*, 112, 08002. EDP Sciences. https://doi.org/10.1051/bioconf/202411208002

Cardeñosa et al. (2025). Integrating portable qPCR and image recognition to combat illegal trade in sharks and rays. *Scientific Reports*, 15(1), 38629. https://doi.org/10.1038/s41598-025-22370-y

Other Fish Species

Damasceno et al. (2016). Molecular identification of Atlantic goliath grouper *Epinephelus itajara* (Lichtenstein, 1822) (Perciformes: Epinephelidae) and related commercial species applying multiplex PCR. *Neotropical Ichthyology*, 14(03), e150128. https://doi.org/10.1590/1982-0224-20150128

Singh et al. (2024). Twenty-three years of PCR-based seafood authentication assay development: What have we learned? *Comprehensive Reviews in Food Science and Food Safety*, 23(4), e13401. https://doi.org/10.1111/1541-4337.13401

Shrimp

Wilwet et al. (2021). Rapid detection of fraudulence in seven commercial shrimp products by species-specific PCR assays. *Food Control*, 124, 107871. https://doi.org/10.1016/j.foodcont.2021.107871

6. FURTHER READING

BELOW ARE SOME SUGGESTED TEXTS FOR ADDITIONAL INFORMATION ABOUT DIFFERENT TAXONOMIC GROUPS AND MOLECULAR TECHNIQUES.

Equines

Wang et al. (2021). A fast PCR test for the simultaneous identification of species and gender in horses, donkeys, mules and hinnies. *Journal of Equine Veterinary Science*, 102, 103458. https://doi.org/10.1016/j.jevs.2021.103458

Pigs

Erwanto et al. (2018). Identification of pig DNA in food products using polymerase chain reaction (PCR) for halal authentication-a review. *International Food Research Journal*, 25(4). (no DOI provided)

Crocodilians

Meganathan et al. (2011). Validation of a multiplex PCR assay for the forensic identification of Indian crocodiles. *Journal of Forensic Sciences*, 56(5), 1241-1244. https://doi.org/10.1111/j.1556-4029.2011.01812.x

Felids

Henger et al. (2023). A new multiplex qPCR assay to detect and differentiate big cat species in the illegal wildlife trade. *Scientific Reports*, 13(1), 9796. https://doi.org/10.1038/s41598-023-36776-z

Techniques

Shivji et al. (2002). Genetic identification of pelagic shark body parts for conservation and trade monitoring. *Conservation Biology*, 16(4), 1036-1047. https://doi.org/10.1046/j.1523-1739.2002.01188.x

6. FURTHER READING

BELOW ARE SOME SUGGESTED TEXTS FOR ADDITIONAL INFORMATION ABOUT DIFFERENT TAXONOMIC GROUPS AND MOLECULAR TECHNIQUES.

Techniques

Tasrip et al. (2019). Loop mediated isothermal amplification; a review on its application and strategy in animal species authentication of meat based food products. *International Food Research Journal*, 26(1). (no DOI provided)

Chaudhary & Kumar (2022). Recent advances in multiplex molecular techniques for meat species identification. *Journal of Food Composition and Analysis*, 110, 104581. https://doi.org/10.1016/j.jfca.2022.104581

Azad et al. (2023). Authentication of meat and meat products using molecular assays: A review. *Journal of Agriculture and Food Research*, 12, 100586. https://doi.org/10.1016/j.jafr.2023.100586

Jenrette et al. (2023). Detecting Mediterranean white sharks with environmental DNA. *Oceanography*, 36(1), 87-89. https://doi.org/10.5670/oceanog.2023.s1.28

Sodré et al. (2024). Molecular identification of sharks from the genus Sphyrna (Elasmobranchii: Chondrichthyes) in Maranhão Coast (Brazil). *Brazilian Journal of Biology*, 84, e274862. https://doi.org/10.1590/1519-6984.274862

Tiktak et al. (2024). Genetic identification of three CITES-listed sharks using a paper-based Lab-on-a-Chip (LOC). PLOS ONE, 19(4), e0300383. https://doi.org/10.1371/journal.pone.0300383

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