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A quick and simple method, usable in the field, for collecting parasites in suitable condition for both morphological and molecular studies

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Abstract

Many methods have been proposed for collecting and fixing parasites, but most were written before the molecular age, and were intended to be practised by experienced parasitologists in well-equipped laboratories. We describe here a very simple method, illustrated by photographs, for collecting helminths from the digestive tract of vertebrates. It only requires a few plastic vials, some ethanol and a means to heat water. Basically, the method consists of: (a) the extraction of all organs from the abdominal cavity; (b) opening the digestive system longitudinally; (c) agitate gut and contents in a saline solution (i.e. ca. 9% NaCl or ¼ sea water in tap water); (d) decant in saline as many times as needed to clean contents; (e) immediately fix parasites in near-boiling saline; (f) discard saline and keep specimens in 95% ethanol. Additional information is given for collecting parasites from fish gills with a similar process. The method will collect most helminths (digeneans, larval cestodes, nematodes, acanthocephalans) from the digestive tract, and monogeneans and isopod and copepod crustaceans from fish gills. The specimens will be suitable for both morphological study and DNA sequencing. The method is simple, fast, inexpensive, and can be used by untrained personnel, even in the field without electricity and without a binocular microscope. It can also be used by trained parasitologists who need to expedite treatment of abundant samples.

Keywords

Method, morphology, DNA, helminth, fish

Introduction

Parasites are one of the most neglected components in evaluations of biodiversity (Bouchet 2006; Poulin 2004; Whittington and Chisholm 2003); however, the simple observation that each free-living animal harbours at least one parasite species immediately suggests that the number of parasite species is at least equal to the number of non-parasitic species (Windsor 1995, 1998) and probably greater than it (Poulin and Morand 2000; Rohde 2001). Parasites are certainly a major component of biodiversity (Cribb and Bray 1998; Dobson et al. 2008; Poulin and Morand 2004) and ecosystems (Kuris et al. 2008; Lafferty et al. 2006) and thus deserve appropriate interest (Brooks and Hoberg 2000; Hoberg et al. 2009; Vannier-Santos and Lenzi 2011). However, parasitology has been often ill-considered (Vickerman 2009) and is still underrepresented in textbooks (Nichols and Gomez 2011). With the progressive decrease in the numbers of parasitologists trained in systematics (Brooks and Hoberg 2001; Cribb 2004; Poulin and Leung 2010) it is likely that further collection of parasites will be done by researchers or technicians who have not been thoroughly trained in parasitological methods. In addition, the development of molecular systematics demands that specimens should be collected in conditions suitable for both morphological and molecular methods; this is not the case for most methods published in classic papers or handbooks.

Most parasitological books, including the oldest ones (e.g. Baylis 1915; Braun and Lühe 1910; Dujardin 1845; Van Beneden 1878, 1887) include a chapter about the processing of parasites. They describe many methods, but agree at least on one point, the need to use only freshly killed hosts to obtain living parasites.

A variety of general methods for the collection and preservation of helminths have been published since the 1950s; methods sometimes varied very much from teams to teams, with controversies arising about the pro and cons of flattening and the use of various chemicals (Berland 1961a, b, 1984; Bray 1984; Burt 1984; Chubb 1962; Cooper 1988; Durette-Desset 1984; Fagerholm 1979; Garvin et al. 1997; Gibson 1979, 1984; Gläser 1965; Gusev 1983; Hanson Pritchard and Kruse 1984; Hargis 1953; Hendrix 1994; Huber 1998; Kennedy 1979; Lichtenfels 1984; Lim 1991; McCue and Thorson 1963; Moravec 1994, 2001; Newman and Cannon 1995; Pritchard and Kruse 1982; Rogers 1966; Unnithan 1957; Williams et al. 1991; Yamaguti 1965a, b, 1968). Various papers have addressed the influence of methods on the measurements of specimens (Bakke 1988; Criscione and Font 2001; Fagerholm 1979; Fagerholm and Lövdahl 1984; Justine 2005, 2007) or the processing of monogenean sclerites (García-Vásquez et al. 2011; Kritsky et al. 1978; Maillard et al. 1982; Malmberg 1957; McDougal and Mizelle 1969; Milne and Avenant-Oldewage 2006; Mo and Appleby 1990; Shinn et al. 1993;

Richards and Chubb 1995; Rohde 1987). The interest in describing new methods is still alive and many recently published works address specific parasites or methods (Albuquerque et al. 2009; Bruno et al. 2006; Cribb et al. 2004; Deveney and Whittington 2001; Galli et al. 2006; Galli et al. 2007; Parker et al. 2010; Snyder and Clopton 2005; Wong et al. 2006).

The emergence of molecular methods has prompted a renewed interest in methods which can process material for both morphological and molecular methods (Harris et al. 1999; Košková et al. 2011; Naem et al. 2010; Strona et al. 2009; Toe et al. 1997; Yoder et al. 2006).

We propose here a method for collecting parasites which can be used by any person who has at least some basic knowledge of vertebrate anatomy, but has no specific training in parasitology. This method modifies Cribb & Bray's "gut wash" method (2010), recently published but in use in various laboratories for years before its publication, and intensively used by one of us (JLJ) on more than 2,000 fish, with molecular (Bray et al. 2009; Černotíková et al. 2011; Miller et al. 2009, 2010a, b; Olson et al. 2010; Perkins et al. 2009) and morphological results (lists in Justine 2010; Justine et al. 2010a, b) published on several parasite groups. Our method requires only access to hot water and alcohol (ethanol) and a few plastic vials and does not require the use of a binocular microscope; it therefore can be practised in the field in harsh conditions, even without electricity. It can be used, also, by trained parasitologists who need to process abundant samples in a limited time. The method is illustrated by photographs. Examples are based on fish, but the method can be used for all vertebrates.

Description of the method

Freshness of material

Parasitological examination should be practised only on freshly killed hosts. "Freshly" usually means hours, but can be extended to 24h if the hosts are kept in a refrigerator. Frozen-thawed material must not be used. Parasites should be alive.

Material and chemicals needed

Material

- Forceps.
- Scissors.
- Several wide mouthed bottles (size related to the size of the fish).
- Plastic Petri dishes or plastic plates (size related to the size of the fish).
- Small or medium-sized plastic vials for storage (as many as fishes × 2 if both intestine and gills are studied).
- Facilities for boiling water (any methods from wood fire to solar apparatuses).

Chemicals

- Ethanol 95%, 20 cl per fish (small species) to more than 1 litre per fish (big species).
- Seawater (for gills of marine fish) and for preparation of saline for gastrointestinal tract (see below). If not available: NaCl.
- Tap water, or any reasonably clean water.

Method for gastrointestinal parasites (Figure 1)

Before processing, prepare saline solution. When near to sea, the best is to mix one part of seawater and three parts of tap water (Cribb and Bray 2010). Filtering is unnecessary. Away from the sea, use 0.9% solution of NaCl in water (9 grams in 1 litre).

Step 1: obtain gastrointestinal tract.

- Open the abdominal cavity, from anus, and anteriorly.
- Cut the digestive system at the level of the anus and oesophagus; if necessary, cut additional attachments.
- Extract all organs and put them on a flat container of appropriate size (a Petri dish for small animals, or a plastic tray).
- Discard liver, spleen and pancreas: keep only the tubular digestive system.
- Untangle the intestine (usually better done with fingers than with metal instruments).

Step 2: open gastrointestinal tract.

- Open the whole digestive system longitudinally (from anus to oesophagus, or vice versa, including stomach) with scissors.
- If pyloric caeca are present, try to open at least some of them
- Open gall bladder.
- Discard large undigested food items, especially those found in stomach.
- Drop entire digestive system into a vial of compatible dimensions (from 20 cm^3 for a small host to several litres to a large one).
- Use saline to rinse the dish on which the digestive system has been open and add rinsing liquid to vial.
- Fill about one third of vial with saline.

Step 3: shake.

- Close vial.
- Vigorously shake vial for about one minute. You should obtain a coloured and unattractive mix.

Step 4: decant and clean.

- Fill vial up to top with saline.
- Allow the contents to settle for about 1-2 minutes.
- Gently incline vial and discard upper three quarters of the mix.
- Check for transparency: if the content is transparent as water, proceed to main step 5.
- If the content is not transparent, fill vial again with saline, wait for settling, and carefully discard three quarters of the liquid. Redo this step as many times as necessary.

Step 5: fix with hot water.

- Gently incline vial and discard almost all liquid, taking care that no solid matter (visible with the naked eye) is discarded.
- Pour near-boiling water in vial, fill.
- Wait at least one minute.
- For small vials and small hosts, continue process in same vial; for large hosts and vials, transfer contents of large vial to a smaller vial which will be used for storage.

Step 6: add ethanol.

- Gently incline vial and discard almost all liquid, still taking care that no solids are discarded.
- Add ethanol up to top of vial.
- The ethanol-solids ratio should be higher than 5:1.

Step 7: label vial.

- Label vial immediately; labels should be hand-written in pencil on tracing paper (waterproof polyester paper is good, but more expensive) and placed within the vial.
- Computer labels are sometimes damaged by ethanol; use only pencil.
- Avoid external labelling of vials with markers; most are erased by alcohol, which can possibly leak. Labels should be *within* the vial. For information on labelling see (Huber 1998).
- The vial should contain a transparent solution, the gut tissue and the label; the gut should not be discarded because some helminths (large digeneans, certain nematodes) remain attached even after the shaking.
- Close vial. You're done.

Method for fish gills (Figure 2)

The method is basically the same but does not require multiple decantations. For freshwater fish, use tap water; for sea fish, use seawater.

Step 1: obtain gills.

- Excise gills by cutting each gill at upper and lower extremity.
- Put all gills in a vial.

Step 2: fix with hot water.

- Pour near-boiling hot water on gills.
- Wait one-two minutes. It is normal if the gills change colour and if the extremities of their filaments curl.

Step 3: add ethanol.

- Gently incline vial and discard most liquid, still watching that no solids are discarded.
- Add ethanol up to top of vial.
- The ethanol-solids ratio should be higher than 5:1.

Step 4: label.

- Same remarks as above (step 7 of gastrointestinal tract)

- The vial should contain the gills, a transparent solution and the label. It is paramount to keep the gills because most monogeneans and copepods will be still attached.
- Close vial. You're done.

Conservation and transport

Optimal preservation of DNA will be attained by keeping the vials in a refrigerator. Vials can simply be kept as they were prepared for years. If the parasitological specialist who performs the triage is elsewhere (often in another continent) the problem arises of how to send vials containing ethanol, which is forbidden by some postal services. It is possible to discard almost all the ethanol to keep only the specimens wet (this also saves weight) without deterioration of the material if the vials are refilled with ethanol as soon as they arrive at their destination.

Discussion

1. The result of a compromise of morphology and molecules

A method suitable for both morphological and molecular studies is obviously the result of a compromise. Formalin fixation (either hot water followed by formalin, or immersion of living specimens in hot formalin) is often considered the best method for obtaining perfect slides of unflattened digeneans (Cribb and Bray 2010), monogeneans (Kritsky and Bakenhaster 2011; Monteiro et al. 2010) and to collect nematodes (Moravec and Justine 2005). However, formalin is not suitable for molecular studies (see below). Freezing at –80° is generally considered one of the best methods to preserve DNA (Nagy 2010), but frozen specimens are usually very bad for morphology (with the exception of monogeneans if only sclerites, not the soft organs, are considered). Also, each parasite group (monogeneans, digeneans, nematodes, acanthocephalans, and also crustaceans) will be best preserved for morphology with some subtle variations; our method is probably not the best for morphology, but will provide specimens which will be acceptable.

2. The need for a solution with correct osmotic pressure

Parasites should be collected and washed in a solution of osmotic pressure close to their original milieu. The use of saline is essential for the collection of gastrointestinal parasites (either for freshwater or seawater

fish); saline solution mimics the osmotic pressure found in the lumen of the intestine. As an example, digeneans are damaged after a few minutes in hypo-osmotic solutions (i.e. tap water) and especially lose tegumental spines, which are important for systematics. The easiest method for the preparation of 0.9% NaCl saline is a mixing of ¼ seawater and ¾ tap water (Cribb and Bray 2010).

External parasites (especially gill parasites) live either in fresh water or seawater, and should be collected in the corresponding milieu.

3. The advantages of washing

One of the advantages of the initial washing and the subsequent successive decantation processes is that the parasites are cleaned of the intestinal mucus: such specimens are particularly well suitable for classical morphological examination, and are also much better for further scanning electron microscope (SEM) examination (Moravec and Justine 2010a, b).

4. Hot fixation vs freezing

Freezing of specimens has been proposed for the collection of monogeneans (e.g. Mizelle 1936, 1938; McDougal and Mizelle 1969), because freezing-thawing does not alter the sclerotised parts of monogeneans, on which much morphological work is based for this group of animals (e.g. Justine and Grugeaud 2010; Pariselle et al. 1991; Rohde 1987). Freezing is certainly an excellent method for DNA processing (Nagy 2010); however, we do not recommend freezing for helminths other than monogeneans because morphological studies (especially of digeneans) require that the body keeps its original shape and proportions; for Bunkley-Williams and Williams (1994), “freezing destroys almost all parasites”.

The advantage of hot water is that it very efficiently relaxes most helminths. Hot water fixation works very well on flatworms (monogeneans, trematodes) (Cribb and Bray 2010; Kritsky and Bakenhaster 2011; Monteiro et al. 2010) and nematodes (Moravec and Justine 2010a, b), and provides acceptable results on some acanthocephalans (unpublished). Parasitic crustaceans on gills, as other arthropods, do not need to be relaxed and fixed in hot water but are still usable if they have been heated. Hot water fixation does not damage DNA (see e.g. Miller et al. 2009, 2010a, b).

The use of our method without the hot water step will result on contracted helminths which will be completely unsuitable for morphological analysis, and should absolutely be avoided.

Other hot fixatives have been used, such as FAA (formalin-acetic-alcohol) (e.g. Kritsky and Fennessy 1999), hot formalin (e.g. Bakke 1988; Rohde and Watson 1985) or hot 70% ethanol (e.g. Berland 1961a) but

manipulation of these hot, ‘steaming’ chemicals is more dangerous than that of hot water because of toxic fumes (formalin, FAA) and possible explosion or flaming (ethanol); hot water is certainly less dangerous, and can be used easily in the field. For the same reason of toxicity, we exclude the use of mercury compounds (i.e. Manter 1926).

5. Ethanol vs formalin and DMSO

Ethanol and formalin were often compared in classical methods, usually with some advantage found for formalin for morphology (Schmidt 1986; Cribb and Bray 2010). However, formalin damages DNA and formalin-fixed material can be used for molecular methods only at the expense of additional and costly effort (e.g. Herniou et al. 1998; Jeon et al. 2011; Klopfleisch et al. 2011; Nadler 1999; Simsek et al. 2011) or is simply useless (Chakraborty et al. 2006). Ethanol has been tested against various other chemicals for the preservation of DNA (e.g. Hajibabaei et al. 2006; King and Porter 2004; Nagy 2010; Post et al. 1993; Quicke et al. 1999; Srinivasan et al. 2002) and is routinely used for DNA studies in helminths (e.g. Shinn et al. 2010).

DMSO (dimethyl sulphoxyde) or DESS (a solution of DMSO, disodium EDTA and saturated NaCl) is considered a good preservative of DNA (Yoder et al. 2006); it is suitable for light microscope morphological studies of nematodes (Naem et al. 2010; Yoder et al. 2006) or monogeneans (Strona et al. 2009) but apparently disrupts morphology in certain arthropods (Frampton et al. 2008). One of the advantages of DESS is that it is not flammable and considered non-toxic and thus allows specimens to be sent by post. However, DESS has not been tested on digeneans. It is more expensive and less easy to obtain than ethanol (Frampton et al. 2008).

We do not recommend acetone, although a good preservative for DNA (Fukatsu 1999), because of its toxicity and unknown effect on morphology of helminths. In addition, acetone dissolves many plastics.

In our method, we recommend ethanol for the following reasons. Ethanol:

(1) Is suitable for molecular methods;

(2) Produces material well suitable for morphological methods;

(3) Is easily found in most countries;

(4) Is of low toxicity and can be manipulated by untrained personnel;

(5) Has a relatively low cost.

The quality of ethanol is paramount for the efficiency of the method, and particularly the quality of further molecular work. Absolute (100%) ethanol is not necessary, because it is very expensive; also, absolute ethanol might contain benzene (Nagy 2010). 95% ethanol solution is the best compromise. However, the manipulator should pay attention to the components of the remaining 5%: it should be water, and no other

solvent. Alcohol sold for flaming purposes has the advantage of a lower cost, but its proportion of ethanol is unknown, and it is usually mixed with methanol (wood alcohol, methyl alcohol) and many other components (to make it unsuitable for human taste) which vary from countries to countries (Lachenmeier et al. 2007); this should be avoided.

Ethanol solutions at 70% or 80% are not acceptable, because ethanol is diluted when added to the remaining of saline (main step 6 of the intestine method, main step 3 of gill method). If only 70% or 80% ethanol (with no other chemical added) is available, it can be used if the material is left overnight in this solution in the labelled vial, then next morning, most of the liquid is discarded and replaced with fresh ethanol solution.

The ethanol-material ratio in the final vial should be higher than 5:1 (Nagy 2010).

6. Flaws of this method

The authors certainly do not pretend that the method will retrieve all parasites. Parasites from special locations, such as trematodes from the scales of fish and others (Cribb and Bray 2010), will not be collected; however, gastrointestinal trematodes represent 95% of fish trematodes (Cribb and Bray 2010). The list of flaws of methods used for parasitological research can be very long: see e.g. (Justine et al. 2010a, b). The shaking part will probably damage most adult cestodes, which will be broken into many proglottids (Cribb and Bray 2010) and thus should not be used for the spiral intestine of sharks or rays; however, the method works very well on most larval cestodes (tetraphyllids), even the tiniest; larval trypanorhynchs in the gut (*Nybelinia*) will be correctly preserved. Larval trypanorhynch cestodes which are in cysts in the abdominal cavity will be collected, if the cysts are kept with the intestinal mass, but probably in suboptimal conditions for morphological study because the tentacles will not be everted (Campbell and Beveridge 1994). Proper collection of trypanorhynchs requires the cysts to be individually open; the cysts can then be added to the gastrointestinal mixture before adding hot water.

However, the method will retrieve, in conditions acceptable for both good morphological and molecular works most flatworms (digeneans, larval cestodes) and nematodes of the gut lumen; these constitute the majority of internal macroparasites found in a vertebrate. Nematodes will be usable for SEM observations (Moravec and Justine 2010a, b). The additional use of the method on fish gills will provide monogeneans in acceptable condition. Gill parasitic crustaceans (mainly copepods and isopods) will also be collected; although heating is generally not necessary nor advised for these groups, the method will provide specimens of acceptable quality; however, not all ectoparasitic crustaceans will be collected by our method, because some easily separate from the fish (Grutter 1995).

7. Separating collection and triage in time and place

Our method described above is mainly based on the "gut wash" method of Cribb and Bray (2010) and resembles the method proposed for monogeneans by Thatcher (2006); however, it differs in that (a) the "wash" obtained after decantation is not immediately examined by the trained parasitologist, but is fixed in hot water and preserved in ethanol for further processing; and (b) it extends the method to all groups of gastrointestinal and gill parasites. Cribb & Bray's method has been intensively used by the senior author over several years, on more than 2,000 fish (Justine 2010; Justine et al. 2010a, b) for the collections of all gastrointestinal parasites (including trematodes, and also all others) and has been found much more time effective than the direct examination of guts; material collected by this method has been forwarded to various specialists of various helminth groups, who were satisfied by the quality of the material, both for morphological (lists of papers in Justine 2010) and molecular studies (Bray et al. 2009; Černotíková et al. 2011; Miller et al. 2009, 2010a, b; Olson et al. 2010; Perkins et al. 2009).

Our method can be practised by a person who is not trained for parasitology, but it requires future processing of the samples by trained parasitologists. Most parasitologists will agree that the triage and collection of living parasites, which are coloured and moving, is easier and more effective and pleasant than that of whitish immobile ethanol-fixed specimens; however, our method separates the two steps of sample collecting, the collection itself and the triage, leaving the second part to trained parasitologists and thus reducing the risk of errors by untrained manipulators. The method can also be easily explained to untrained technicians, and it is hoped that the present step-by-step explanation and photographs will make it easily understandable by a reader untrained in parasitology. Finally, it can be practiced in remote exotic places without modern comfort (which are usually the places in the world where biodiversity is at its greatest), while further processing can be done in well-equipped laboratories.

Another possible use of our method is to treat abundant material in a limited time. Pritchard and Kruse (1982), wrote "the collector must be ready to work hard and long". However, there are limits to human stamina, even for the most trained and motivated parasitologists, when they receive at the same time more freshly killed hosts specimens than that they can process while they are still fresh: in such cases, our method can be an alternative to the classical collection of living parasites.

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Figure Legends

Figure 1: Collecting helminths from the gastrointestinal tract.

A. Open fish from anus.

B. Open abdominal cavity and extract all organs. Discard liver.

C. Moisten organs with saline.

D. Open stomach longitudinally.

E. Open entire intestine longitudinally.

F. Drop entire digestive system in a container and close it.

G. Vigorously shake container. Allow decantation for 1-2 minutes.

H. Discard upper part. Repeat until remaining liquid is transparent.

I. Add near-boiling water. Discard most of it.

J. Fill vial with ethanol.

K. Add internal label in vial.

Figure 2. Collecting gills with helminths and other parasites.

A, B. Cut each gill at both extremities.

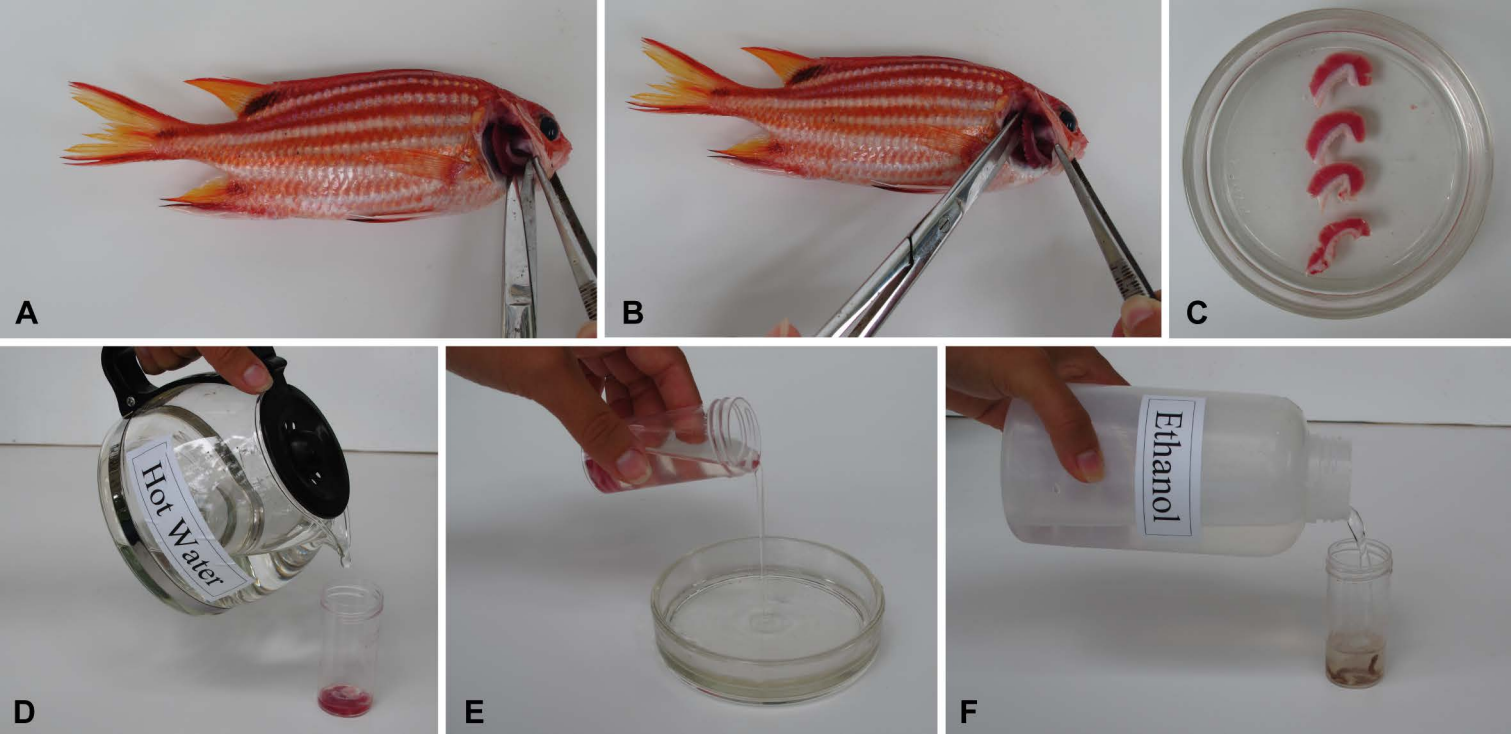
C. Collect gills in sea water (for sea fish) or tap water (for freshwater fish).

D. Put gills in container and add near-boiling water.

E. Discard most water.

F. Fill vial with ethanol. Add internal label in vial.


A
B
Saline
C
D
E
F
Shake !
G
H
Hot Water
I
Ethanol
J
K


A
B
C
Hot Water
D
E
Ethanol
F