

Project TigrisID detailed description

Any samples (from inspected tigers in captivity, wild tigers, suspicious seized products...) are welcome. Reference samples can include **tissue** (e.g. collected by biopsy darts), **hair**, **saliva**, **blood or faecal samples (droppings)**. The research team will provide guidelines and a **protocol for the sampling** and shipment of the reference material from abroad. Testing of products potentially containing biological material of *Panthera tigris* (e.g. broth, paste) for purposes of investigation carried out by partner foreign authorities will be also possible.

The output of the project should consist of the following products:

**TigrisQuant** - RT-PCR assay for specific quantitation of minute amounts of *Panthera tigris* DNA in heavy-processed products. The research team plans to employ the testing of mtDNA which is more abundant than nuclear DNA and also less prone to degradation. TigrisQuant should identify the presence of *Panthera tigris* DNA exclude falsely positive results from non-CITES cats and detect the presence of PCR inhibitors using internal amplification control.

**TigrisPlex** - STR multimix for individual identification of *Panthera tigris*. The research team plans to select tetranucleotide STRs, test their discrimination power (polymorphic), robustness and ability for multiplexing. Resulting multiplex(es) will enable to generate DNA profile suitable for sample-individual comparison or kinship analysis.

**TigrisBase** - database for storage and comparison of *Panthera tigris* DNA profiles obtained from forensic, inspection and reference samples. The database will provide a similar functionality as CODIS database used by the law enforcement agencies in the field of human identifications.

The chemistries used for TigrisQuant and TigrisPlex are commonly used on a standard laboratory equipment (RT-PCR, capillary electrophoresis) and there will be no technical obstacles to deploy the products outside the Czech Republic.

All methods and procedures of the project will follow the ISFG recommendations for animal DNA testing - mainly tetranucleotide STRs, population database, species specific assays, sensitivity, compatibility with current forensic techniques (RT-PCR, capillary electrophoresis, massive parallel sequencing...).



TIGER GENETICS

Development of Diagnostic Tools for DNA Analysis Based on Individual Identifications and Species Identification in Processed Products



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Background

Trade in tiger parts and products seems to be very extensive not only in Asia but also in Europe due to high demand in Asian communities living outside Asia. There are many live tigers kept in various facilities and some of these facilities are probably used as a source of products for black market and are involved in illicit trade. There has been a significant increase in tiger product seizures in the Czech Republic over the past years. The trade does not just involve bones, claws, teeth and skins as it is referred by enforcement authorities worldwide. New unknown product types appear - broth, paste, powder, wine... that are difficult to identify. This trade has unfortunately been more extensive than it has been expected.

Table 1: International Society for Forensic Genetics recommendations regarding the use of non-human (animal) DNA in forensic genetic investigations:

1	The same procedures to ensure integrity and traceability of the items should be employed in the collection and examination of animal samples as undertaken for any other forensic investigation.
2	Validation studies from non-domesticated species should use voucher specimens where possible. If this is not possible then a justification needs to be made for the sample type used.
3	The choice of locus/loci used in species identification, such as, but not restricted to, the mitochondrial genes cyt b, COI, and the D-loop region, needs to be justified based on the ability to identify the unknown species among those that are close genetic relatives.
4	The nucleotide sequence and map showing the location of the primers used in species testing needs to be provided or referenced to a previously published article.
5	Intraspecies and interspecies studies should be provided for any novel primer set used in species identification. The process undertaken to validate the test should be provided, including, but not exclusively, studies on sensitivity, specificity, reproducibility and mixed samples.
6	Primers used to amplify polymorphic DNA should be tested to ensure specificity and reproducibility and should be published in the public domain.
7	If repeat-based polymorphic loci are used for individualization, tetrameric short tandem repeat systems should be used preferentially.
8	Sequenced allelic ladders are essential for the accurate designation of alleles and should be used in all STR typing. The number of repeats should be the basis of reporting of results rather than using only the size based on the number of base pairs of any samples tested.
9	In relationship testing, the mutation probabilities of the STR alleles should be estimated if encountered, or at least the probability of a mutational event occurring should be considered when there is genetic inconsistency at a single or few loci while all other loci show genetic consistency.
10	Relevant population and forensic genetic parameters including allele frequencies should be estimated.
11	A kinship factor should be determined and applied in any calculation. The type of kinship factor applied should be stated clearly and justification should be made for the factor incorporated.
12	A comprehensive casefile should be maintained. A likelihood ratio approach is the recommended way to evaluate the weight of the evidence, considering more than one proposition.
13	Accreditation should be sought if DNA testing of non-human animal DNA for a particular purpose is to become routine.

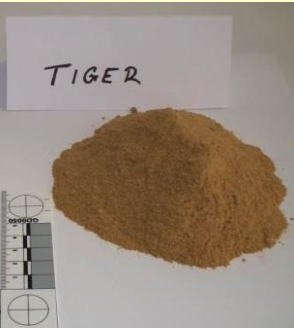
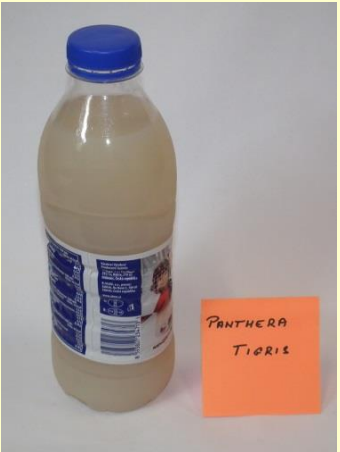
References:

- <sup>1</sup> Votrubova, Jitka, et al. "Operation Tiger's Eye: DNA testing of traditional Chinese medicine artifacts in the Czech Republic." *Forensic Science International: Genetics Supplement Series* (2017).
- <sup>2</sup> Linacre A, Gusmao L, Hecht W, Hellmann AP, Mayr WR, Parson W, et al. ISFG: recommendations regarding the use of non-human (animal) DNA in forensic genetic investigations. *Forensic science international Genetics*. 2011;5(5):501-5. doi: 10.1016/j.fsigen.2010.10.017. PubMed PMID: 21106449.



Fig. 1-3: Several complete raw tiger skeletons smuggled from Prague to Hanoi were seized at the Prague airport (hidden in hi-fi speakers). Frequent seizures are also claws and teeth as jewellery for happiness.

Fig. 4-8: Many bottles with suspicious liquid, cubes of strange matter and sacks with powder were seized during Operation Osseus and Operation Tiger Eye at the Prague airport. DNA of *Panthera tigris* was found in numerous of these products<sup>1</sup>.





## Inspections of tigers:

CEI has resolved to **inspect all tigers kept in captivity** in the Czech Republic (CR) and take their DNA samples. Totally **404 tigers** were recorded in the CR in the last years, but only 130 of them were still alive and present in the CR in 2017. Most of the tigers are in private facilities, only 40 in zoos. CEI has so far **collected 180 genetic samples** - 136 samples of hair and 44 faecal samples.

Many tigers have been traded and transported to or from the CR within the EU (71 tigers since 2015), some tigers were exported from the CR outside the EU, even to Asian countries as Vietnam or Thailand (totally 57 tigers since 2006).

Many discrepancies in records were found including **suspicious deaths and missing tigers**. Alleged high mortality of adults and tiger cubs was found – normal life expectancy for tigers (both in the wild and in zoos) is about 20 years, tigers in private facilities in the CR die at the average age of 5 years. There is no legal obligation for breeders to keep official documentation confirming disposal of dead bodies.

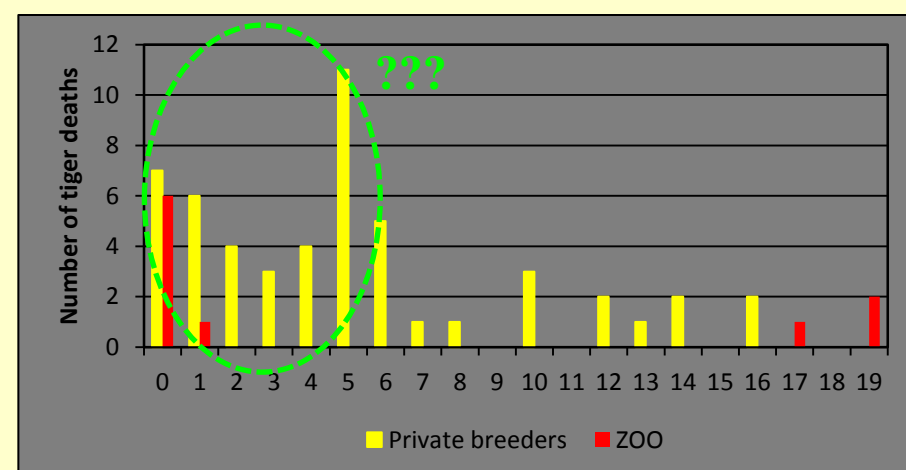


Fig. 9: Tiger inspections done in the CR

Fig. 10: Number of died tigers at a certain age

In connection with the increasing number of seized tiger products it is assumed some breeders may be involved in the illegal trade in tiger products organized most likely by persons from the Vietnamese community living in the CR.

## Why is there a need to deal with tiger genetics?

There are numerous scientific papers on DNA identification of *Panthera tigris* species but unfortunately the published studies do not fully follow the recommendations of ISFG (International Society for Forensic Genetics) for non-human DNA typing<sup>2</sup> (see Table 1).

Identification of tiger DNA in **heavy-processed materials such as broth or paste is extremely difficult** because DNA is often degraded due to the long boiling used in product preparation and sometimes mixed with genetic material of other species, including plants. Investigation of these cases is complicated and often unsuccessful because the key evidence (presence of tiger DNA) is impossible to gain.

**Individual tiger identification** seems to be the only way how to trace the source animals and producers. However such profiling is currently not feasible since no individual markers are known and tested.

Hence enforcement authorities have no tools and means how to match seized tiger products with suppliers.

The Czech Republic has currently approved a new research project focused on tiger genetics that has been planned to last for 4 years. The main resources for the research will be covered by the grant of the Czech Ministry of Interior. The project and its results should help to reduce national and international crime with *Panthera tigris* parts and products.



Fig. 11: A tiger held in private facility in the CR

## Project TigrisID

The aim of the project is to **develop novel tools for DNA based identification** of biological material of *Panthera tigris* species. Major components of the project are:

**TigrisQuant** - to develop and validate methods for species identification of *Panthera tigris* in heavy-processed material as broths, tiger paste, wine, boiled bones etc.

**TigrisPlex** - to develop STR kit(s) for individual identification of tigers (multimix for simultaneous amplification of informative STR loci)

**TigrisBase** - creating a database for storage and comparison of DNA profiles from reference, inspection and unknown forensic samples of *Panthera tigris*

## TigrisID

**TigrisQuant**

- RT-PCR assay for specific quantitation of *Panthera tigris* DNA

**TigrisPlex**

- STR based individual identification of *Panthera tigris*

**TigrisBase**

- Database for *Panthera Tigris* DNA profiles

**Illegal trade is not confined to state borders - a global approach is important!**

## How other countries can be involved?

The problem of heavy-processed products probably containing tiger DNA is not just about the CR. For example bottles with strange liquid were found during the raid in Tiger Temple in Thailand in 2016.

The key point of the project is an **accurate population study** needed for the kinship calculations. The higher number of DNA profiles from unrelated individuals will result in more precise population data of used STRs and thus more precise statistical calculations (confirmation of database hit between the stored profile and unknown forensic samples).

In order to address the mutation rate of the selected STR markers the research team need to obtain approximately 100 samples from unrelated individuals.

**The input of Interpol or Europol** for the project is based on the position as **leaders of international enforcement collaboration**. Although the research will be carried out by the Czech Republic it would nevertheless be more effective if information, experience and results will be shared with other countries.

The aim of suggested activity is **collaboration** with tiger range and consuming countries as well as with countries where tigers are held in captivity, with their authorities and forensic laboratories especially for the purposes of **obtaining other samples**. Samples will be used in the research (population testing, statistical evaluation of mutation rate done by ISFG recommendations) and consequently added to the **TigrisBase**. The database is being created by the Czech Republic based on the Czech collected samples but can be easily extended to an international database in future (for example similar to RhODIS database focused on rhinos and led by University of Pretoria).



Fig. 12: The Tiger Temple raid - seized dead animals and products (Thailand, 2016)



Fig. 13: Sample of tiger hair collected in the CR

Sharing of DNA samples between countries (enforcement authorities, labs) could be limited by certain restrictions by CITES or national rules so an official platform for collaboration would be very appreciated. Moreover, **mutual agreements about collaboration and sharing tiger DNA samples** can be signed by authorities of concerned countries.

The subsequent goal is **sharing experience and providing results of the genetic research to other countries and relevant authorities**. The research team expects to publish results in scientific and forensic journals so that they can be widely used by forensic and enforcement authorities.



# Tigris ID: Guidelines and Sampling Protocols



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## Background:

This document builds on the internationally distributed material representing the Czech project Tigris ID which aim is to **develop novel tools for DNA based identification** of biological material of *Panthera tigris*. Major components of the projects are:

- ✓ to develop and validate methods for species identification of *Panthera tigris* in heavy-processed material as broths, tiger paste, wine, boiled bones etc.
- ✓ to develop STR kit(s) for the individual identification of tigers (multiplex for simultaneous amplification of informative tetranucleotide STR loci)
- ✓ to create a database for the storage and comparison of DNA profiles from reference, inspection and unknown forensic samples of *Panthera tigris*.



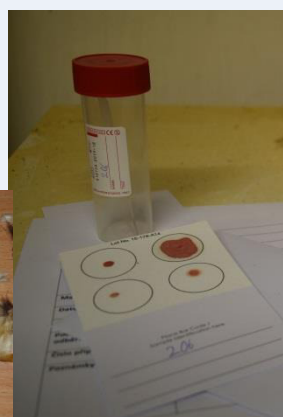
The key point of the project is an **accurate population study** needed for kinship calculations and selection of informative STR markers and thus it is necessary to analyse the genetic material from as many unrelated tigers as possible. **The collection of any genetic samples of tigers and their shipment to the Czech Republic** is therefore very welcome. Although the research will be carried out by the Czech Republic, it would nevertheless be more effective if the information, experience and results will be shared with other countries. The aim of this project is collaboration with tiger range and consuming countries as well as with countries where tigers are held in captivity. **Mutual agreements about collaboration, sharing tiger DNA samples** and subsequently sharing the **results of genetic research** can be signed by authorities of concerned countries. The database of tiger DNA profiles can be easily extended to an international database in future.

## Types of samples:

Any samples from tigers in captivity, wild tigers, suspiciously seized products potentially containing biological material of *Panthera tigris* (e.g. broth, paste) etc., are welcome.

Requested samples from *Panthera tigris* specimens can include just one or a combination of the following materials:

- blood
- saliva
- hair
- soft tissue
- hard tissue (tooth, bone)
- faecal samples (droppings).



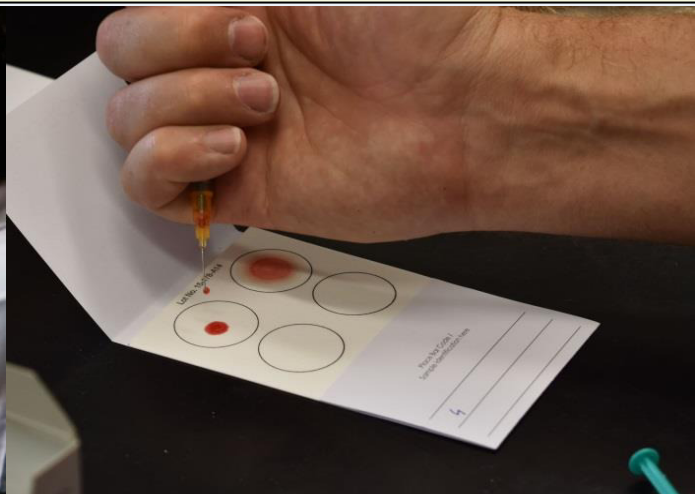
From live animals, **samples of blood, saliva, tissue or hair plugged with bulbs** are preferred. If the collection of these samples is not possible, samples of hair found in the environment or animal droppings can be collected. In the case of collection from the bodies of dead animals, it is possible to collect all types of the above-mentioned samples. Depending on the degree of body decomposition, it is preferable to collect blood or tissue samples as well as tooth samples in the case of skeletal remains.

Since the material is to be used for genetic analysis, it should be minimally contaminated by the collector during collection. The collection of any sample should be carried out with disposable **rubber gloves**. The gloves should be changed before each new collection of samples.

All samples collected must be **properly marked** and accompanied with a **detailed information sheet**. In the case of animals kept in captivity, it is appropriate to indicate at the minimum the country of collection, breeding facility and the collection day. Ideally, also the sex of the animal and its identification number (microchip), if you are confident that the sample comes from that specific animal. In the case of a non-invasive collection of hair or faeces in enclosures with multiple animals, all of the animals present should be mentioned. It is also appropriate to **indicate any possible family relations** between the animals whose samples you are sending. A simple solution is to mark the sample with a number and print a breeder's card with all of the available data. For wild animals, at a minimum the country of origin, the coordinates of the place where the specimen was collected, and the sampling date must be recorded. The more information you provide us about the sample, the better.

Blood collection may only be performed by a veterinarian or a person authorized to do so. Blood samples for genetic purposes can be processed in both liquid and dry states. However, **dry samples are more suitable for long haul transport**. After the animal's vein has been pierced and blood drawn, the blood drops are transferred on the paper storage cards. The diameter of the blood stain should be **at least 2 cm**. Prior to storing the paper storage card with the sample in a paper envelope, please wait until the blood sample has **completely dried**. If this procedure is not possible, put the blood into the EDTA collection tube.

- **Tools - injection needles, filter paper for blood collection, paper envelope, microEDTA collection tube**



Some tigers (especially those who have a good relationship with the zoo keeper and are not afraid to come to the grid) can be offered a cotton/synthetic swab through the bars which the tiger will take to the mouth. This collection can only be carried out by the tiger's zoo keeper. Samples of saliva can also be taken from an anesthetized animal. The swabs should then be placed in a paper box (sampling set).

- **Tools:** sampling set - cotton/synthetic swab, paper box (a prepared box that can be folded on site)







### 3. Tissue collection from a dead animal

In the case of a dead animal, tissue sampling is very simple and can be done by anyone. A sterile knife/scalpel is sufficient to collect a part of the soft tissue from the animal (for example, a part of the ear 0.5 cm<sup>2</sup>) and put it into a sterile, sealable tube filled with 96% alcohol.

The collection of sample can also be made from a taxidermized or prepared-to-be-taxidermized part of the tiger body that is available (skin, tooth, claw, bone). If some chemical reagents have already been applied, it is advisable to make a note of it on the sample. The packaging of such samples is described in the chapter on storage.



- **Tools: rubber gloves, sterile knife/scalpel, sterile sealable tube, 96% ethanol or isopropanol**



### 4. Tissue collection from the living animal

A live animal tissue sample can be collected in two ways, either when the animal is fully conscious or during immobilization. However, in both cases, the collected sample should only be carried out by an experienced veterinarian or a person authorized to do so.

**Collection when the animal is fully conscious** is possible using **biopsy darts** both in captive animals and in wild animals. These darts are similar to those used with immobilized animals from a distance. It is necessary to always use a sterilized dart. More information is available here: <http://www.globalsupplies.co.za/index.php/pneu-darts/biopsy>. The dart is fired at the animal and when it penetrates the skin, it collects a sample but then drops off the tiger's body. Due to the distinctive colour of the darts rear end, it is easy to track it. It is then possible to unscrew the part of the dart covering the needle with the sample of the skin. The sample should be removed with sterile tweezers and inserted into a sterile, sealable micro tube filled with alcohol.



- **Tools: biopsy darts, dart gun, sterile tweezers, sterile sealable micro tube, 96% alcohol**

**If the animal is anesthetized**, it is possible to collect a sample using biopsy punches. Their use is the same as darts. However, the difference is that the sample is collected by being manually pushed into skin. More information is available here: <https://www.wpiinc.com/product-listers/biopsy-punches/>.



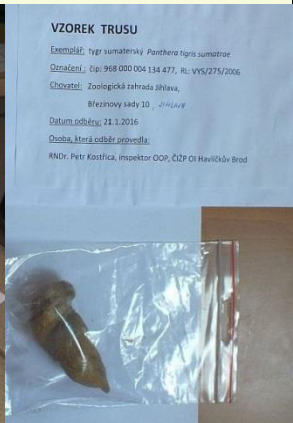
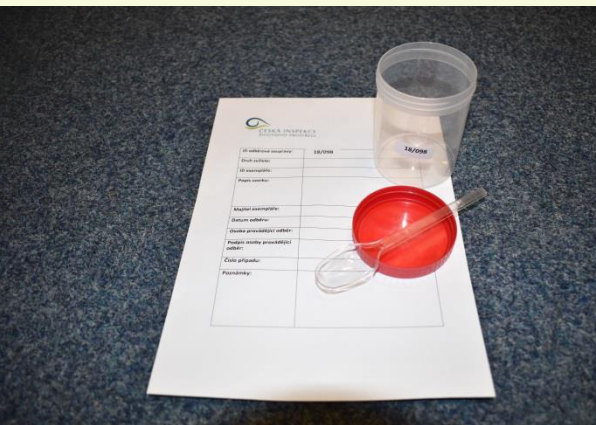
- **Tools: biopsy punches, sterile tweezers, sterile sealable micro tube, 96% ethanol or isopropanol**



### 5. Droppings collection

The sampling can be done by keepers during cage cleaning. The collected sample should be a size of walnut. For DNA analysis **intestinal epithelial cells** are important, which are **on the surface of the droppings (not inside)**. Therefore, it is necessary to collect the sample as much as possible from the surface of the droppings (not from the inside) or collect the whole dropping with a removable plastic bag turned inside out (as it is done when picking up after dogs). When using a tool, the collection must always be done with a clean tool - for example, with a disposable plastic spoon that is discarded after use to avoid contamination of the DNA sample. Place the droppings sample into a clean, sealable plastic cup, box or a heavy-duty plastic bag.

- **Tools: plastic bag/collecting container, collecting tool – e.g. disposable plastic catering supplies or gloves**

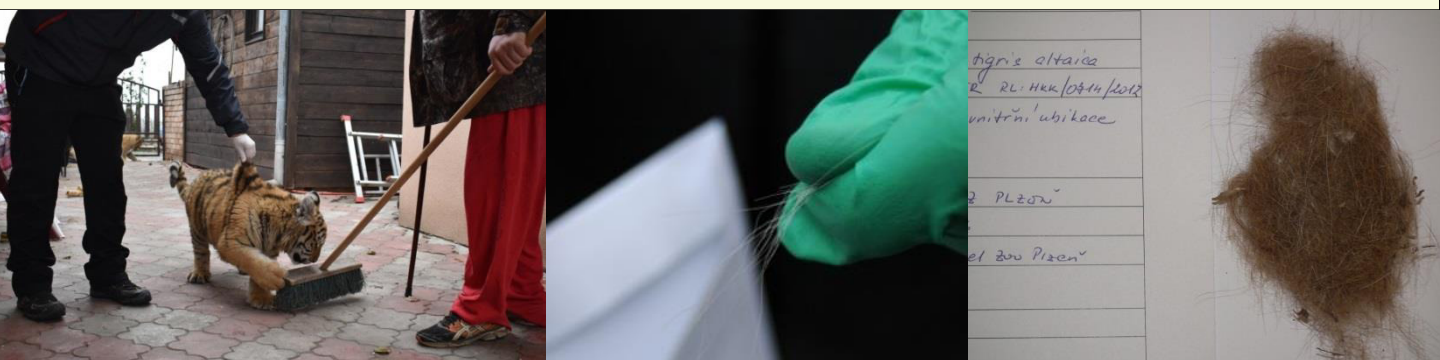




## 6. Hair collection

The collection of the hair sample can be done invasively as well as non-invasively. However, in both cases, it is necessary for the hair to contain bulbs of hair. In case of invasive collection, the hair samples are pulled straight out of the animal with, for example, tweezers or a rubber gloved hand through the bars. In all cases, it is up to the zoo keeper to determine from which animal the collection of a sample is possible and perform the collection himself. This is usually possible for tigers trained for the circus, tigers raised by people like pet animals and tiger cubs taken from their mother. This collection is of course also possible for immobilized animals. The advantage of invasive collection is the certainty that the sample comes from specific animal and is freshly collected. Non-invasive sampling is done through collection of hair samples in places where the tigers scratch or rub. The hair is often trapped on bunks or bars and can be collected and put into a paper envelope (not in a plastic bag where DNA can degrade). The disposable gloves (gloves must be replaced between each collection of samples) or tweezers (between each collection of samples it is necessary to remove all hair or sterilize it with alcohol or fire which is even better) may be used.

### ➤ Tools: tweezers/rubber gloves, paper envelope



### The storing and shipping of the samples:

#### ➤ Blood samples

As already mentioned in the section on collecting of blood samples, wait until the sample of blood has dried completely before placing the filter paper with the sample in the paper envelope and shipping. It is always advisable to keep these samples in a dry place to prevent humidity. If samples are left in a liquid state, it is advisable to keep the blood collected in the micro-tubes frozen at -20 °C. Blood in a liquid state can be sent only in the special transport medium (DNA shield) – please contact Forensic DNA service before such shipping.

#### ➤ Saliva samples

The sample of saliva on the cotton swabs should be stored in a paper box pervious to air (sampling set) - humidity in a plastic or closed plastic box must be prevented. The paper box with the swab can be sent in a paper envelope.

#### ➤ Tissue samples

Small tissue samples placed in sealable microtubes and treated with alcohol are suitable to store frozen at -20° C until shipping. Larger samples collected from dead animals can be treated with salt and stored frozen at -20° C. Samples of taxidermized body parts can be stored in paper envelopes, plastic boxes or frozen if taxidermy has not been completed (prevent the formation of humidity in the plastic bags containing samples).

#### ➤ Droppings sample

If a fresh, warm dropping is collected, it should be allowed to naturally cool. It is then necessary to freeze it as soon as possible and it is the best to keep it cool until placing it in a freezer. It is advisable to prepare in advance frozen cooling inserts or ice-bags placed into thermobags or thermoboxes together with droppings packed in plastic bags/collection containers. Avoid the smudging or wetting of paper labels with the description. It is advisable to mark the samples with permanent markers or use an ordinary pencil to write on paper labels. Samples should be stored at -20° C until shipping. Please contact Forensic DNA service before shipping.

#### ➤ Hair samples

Unlike droppings, keep hair dry in a paper envelope (not in a plastic bag) and at room temperature (do not freeze, moisture leads to DNA degradation). Send them in a paper envelope.

The sharing of DNA samples of tigers between countries can be **limited by certain restrictions**, e.g. CITES or national rules. The sending within EU should be without problems, **CITES permits** are necessary for the sending from non-EU countries. Please contact us before shipping any samples you have collected.

### References:

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