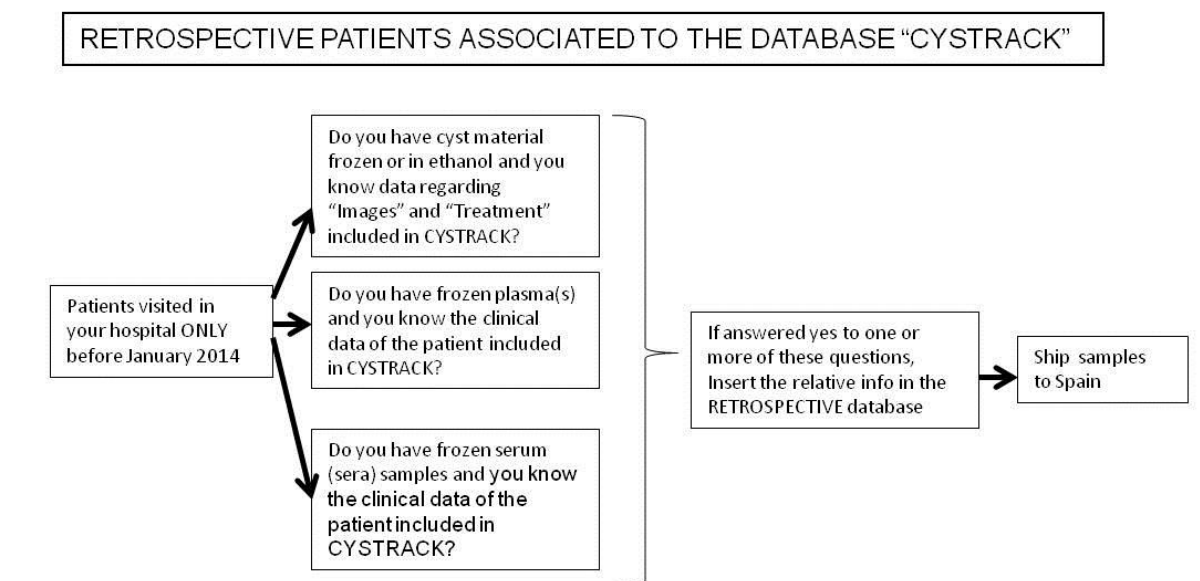
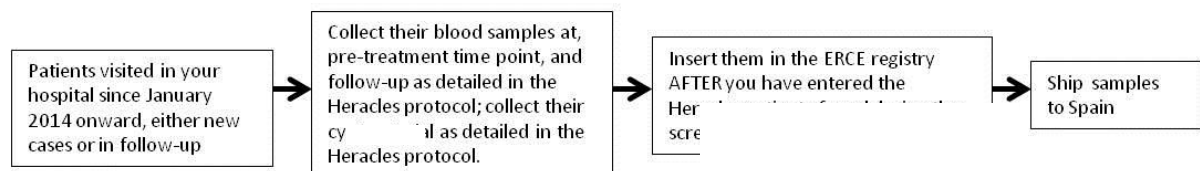
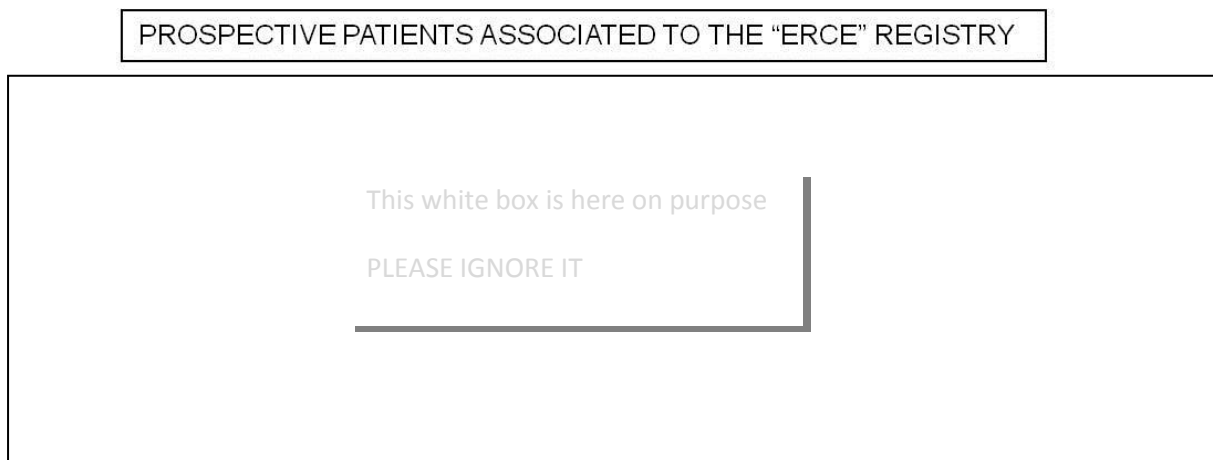


BIOLOGICAL SAMPLES PROCESSING AND LABELLING

1. CLASSIFICATION OF PATIENTS TYPES AND SAMPLES TO COLLECT

This graph explains the patients types, their registration in the databases, and the samples we are collecting from them. A detailed explanation is provided in the next pages. Ignore the white box below, please



2. “Prospective patients” = patients that you see in your hospital from January 2014, either new cases or at follow-up.

- They will be entered in ERCE Registry once they are visited
- They will be assigned a progressive ID code automatically by ERCE Registry
- Please try to enter as more detailed data as possible, in particular the past medical history. You can ask the patient to bring the clinical records on a next visit, to complete the ERCE registry data.

- IF YOU DO NOT HAVE ANY SAMPLE STOCKED FROM THEM, you collect their samples according to the Heracles protocol from now on

- IF YOU HAVE ALREADY SAMPLES FROM THEM taken during previous visits, ATTENTION, PLEASE, for any of these “follow-up” patients, the eligibility criteria for shipping them to Spain are:
 - cyst material is appropriately preserved (frozen or in ethanol are both acceptable), and at least the information on cyst stage and treatment is available.
 - plasma and sera are frozen, and complete medical history is available.

- You will have to take from them a written informed consent to the use of biological material
- You will have samples coded as follows
P (for “Prospective”) - ID code give by ERCE - sample code – date – type of material - preservative

- Sample code is a CONSECUTIVE NUMBER
 - for consecutive time points of collection for serum, plasma (buffy coat, plasma in DMSO and blood culture supernatant)
 - for different cysts or cyst materials (membranes, protoscoleces, hydatid fluid) from the same patient. In ERCE you will see that all cysts are assigned a cyst progressive number. Please indicate this “Cyst number” as the sample code on the vials

- Examples for patient number 645 (number 645 comes out from ERCE registration of the patient)
 - Serum samples at consecutive time points are:
 - P-645 - 1 – dd/mm/yyyy – serum
 - P-645 –2 – dd/mm/yyyy – serum

 - 2 Cyst operated by surgery may be:
 - P-645 – cyst 1– dd/mm/yyyy – membranes – Frozen
 - P-645 – cyst 2 – dd/mm/yyyy – cyst fluid - frozen
 - P-645 – cyst 2 – dd/mm/yyyy – PSC – PFA
 - P-645 – cyst 2 – dd/mm/yyyy – PSC – Karn.
 - P-645 – cyst 2– dd/mm/yyyy – membranes – frozen

3. *“Retrospective patients” = patients (and samples) that you have seen in the past in your hospital and that you have lost to follow-up*

- ✓ They will NOT be entered in ERCE (unless they show up in 2014 or later; if this happens you’ll follow the procedure for “Prospective patients” above)



ATTENTION! Retrospective samples of sera and cysts from patients can be used in a retrospective study, **ONLY IF** they were properly stored and are associated with the relevant patients information detailed below that should be entered in the retrospective database “CYSTRACK”

REQUIREMENTS TO SEND RETROSPECTIVE SAMPLES ARE:

- You have from the patients a written informed consent to the use of their biological material
 - If you have serum or plasma from these patients, the clinical data of the patient detailed in CYSTRACK is available (see below). If you have cyst material, the information about “Images” and “Treatment” detailed in CYSTRACK is available (see below).
 - At least 0.5 ml are available for each serum sample and for plasma samples.
 - Sera and plasma have been stored at -20°C or below.
 - When cyst material from more than 1 cyst per patient is available, material from single cysts should have been stored individually (no pooling of different cysts from same patient) with membranes stored in 70% ethanol and/or frozen; cyst fluids must have been stored at -20°C or below; protoscoleces must be in preservative or frozen.
- ✓ It is not required that you have ALL above material from every patient. You can give only one material type or multiple material types from every patient, provided the requirement for each material are fulfilled.
 - ✓ You have to start a database of these samples (in a book or in an excel sheet) and to assign each patient a progressive “story number”, starting with number 1.
 - ✓ Samples will have to be coded: you have to introduce your own code of your laboratory in the database in this box in the RETROSPECTIVE DATABASE CYSTRACK. This code will be mark in the tubes when you send us them to the biobank. In this way the traceability is sure. Later Cystrack will give an automatically code to each sample.


Create

Samples

Patient *  

Date *

Type *

Store Code 

You will have to enter these retrospective patients in the RETROSPECTIVE DATABASE CYSTRACK. This can be accessed at <http://cystrack.irnasa.csic.es/login>. The database is available in English and Spanish. Each user will receive a username and a password to enter patients’ data.

Please, check if your data are complete before entering them into the database.

Patient

Country *

Postal Code *

History No. *

Birth Year * Pediatric

Autochthonous case Birth Country

+ Previous Cystic Echinococcosis

Clinical Data

Symptoms New Case Surgery Relapse

Relapse after other treatments Persistence Residual Cavity

Sera collection and serology (please also use this section for patients in follow-up)

Delete	Date	Serology	Technique	Antigen	Specify antigen	Titre
<input type="checkbox"/>	<input type="text"/>	Positive	Choose a Technique	Choose a Antigen	<input type="text"/>	<input type="text"/>



Images (please also use this section for patients in follow-up)

Delete	Date	# Cyst	Technique	Specify technique	WHO Classification	Size	Location	Specify location	Complications	Image file
<input type="checkbox"/>	<input type="text"/>	<input type="text"/>	Choose a Technique	<input type="text"/>	Choose a WHO Class	<input type="radio"/> ≤3cm <input type="radio"/> >3cm	Choose a Location	<input type="text"/>	<input type="checkbox"/>	<input type="text"/> <input type="button" value="Examiner"/>



Treatments ("Clinical Management")

Delete	Type	Specify treatment	Start Date	End Date	Drug	Specify drug	Dose (mg/kg/day)	Complications
<input type="checkbox"/>	Choose a Treatment	<input type="text"/>	<input type="text"/>	<input type="text"/>	Choose a Drug	<input type="text"/>	<input type="text"/>	<input type="checkbox"/>



4. ***REAGENTS CHECK LIST***

- ✓ PBS pH 7.2
- ✓ NaOH 1M (40g powder per 1 litre)
- ✓ DMSO

- ✓ Paraformaldehyde 4%
 - 40 g Paraformaldehyde powder in 1 litre PBS
 - Heat at 40°C (ATTENTION! No above 65°C!!!) while stirring to dissolve
 - Add 2 ml 1M NaOH (the solution should clarify)
 - Filter
 - Divide in 2 ml aliquots and store at -20°C

- ✓ Karnowsky
 - Prepare Cacodylate buffer 0.2M pH 7.4-7.6:
 - A) 10.7 g Sodium cacodylate $\text{Na}(\text{CH}_3)_2\text{AsO}_2 \cdot 3\text{H}_2\text{O}$ in 250 ml distilled water
 - B) 4.14 ml HCl (36-38%) in 250 ml distilled waterFinal solution: mix 50 ml solution A with 4.2 ml solution B

 - Prepare the Karnowsky medium as follows:
 - 25 ml Paraformaldehyde 8% (2 g paraformaldehyde powder in 25 ml distilled water → heat at 40°C while stirring to dissolve → add 1-3 drops of 1M NaOH to clarify)
 - 10 ml glutaraldehyde 25%
 - 15 ml Cacodylate buffer 0.2M pH 7.4-7.6
 - 0.025 g anhydrous CaCl_2

 - *Divide in 1 ml aliquots and store at -20°C*

- ✓ Ethanol 70%

- ✓ Ethanol 50% in PBS

- ✓ OTHER MATERIAL
 - filters with 0.45 μm pores

5. BLOOD SAMPLES

Empty tubes for serum

- Leave tube standing for 2-6 h at room temperature
- Centrifuge at 5000 rpm for 10 min (if possible. Otherwise standing for 6h should be enough)
- Collect serum
- LABEL each vial with patient ID code as explained before
- Store at -80°C as soon as possible (if needed serum can be stored at +4°C for up to 1 week)
-

K-EDTA tubes for plasma and buffy coat

- Process samples within 6 hours. Maintain blood at +4°C until processed
- Centrifuge tubes (10 ml blood in total) at 1500 rpm for 10 min
- Collect plasma and pool together in 1 tube
- Collect buffy coat and pool together in 1 tube
- LABEL each vial with patient ID code as explained before
- Store at -80°C

K-EDTA tubes for plasma (with and without DMSO)

- Process samples within 6 hours. Maintain blood at +4°C until processed

5 ml FOR DMSO STORAGE

- Centrifuge blood (5 ml) at 600g for 15 minutes at +4°C
- Collect the supernatant (plasma) in a clean tube and discard the pellet
- Centrifuge the supernatant (plasma) at 1500g for 15 minutes at +4°C
- Collect the supernatant (plasma) in a clean tube and discard the pellet
- Centrifuge the supernatant(plasma) at 10000g for 15 minutes at +4°C
- Add DMSO in a 5% volume
- LABEL each vial with patient ID code as explained before
- Freeze at -80°C

5 ml without DMSO

- Centrifuge blood (5 ml) at 1500g for 15 minutes at +4°C
- Aliquot the supernatant (plasma) in 2.5 Eppendorf tubes, each aliquot consisting of at least 200 μ l
- LABEL each vial with patient ID code
- Freeze at -80°C

6. *PARASITIC MATERIAL*

HYDATID CYST FLUID (HCF)

- Collect HCF by percutaneous puncture or during surgery in a sterile manner in a 50 ml falcon tube
- Collect HCF when this can be easily collected; do not collect it if surgery is performed on CE3b cysts or when the fluid is very difficult to obtain in multivesiculated cysts
- If more than one cyst is aspirated, please process material from different cyst separately cyst by cyst and give cyst number code on the stored material (see cyst number code on case report form)
- Defrost 1 aliquot of Paraformaldehyde 4% and 1 aliquot of Karnowsky
- Divide the HCF in n = 3 15 ml Falcon tubes
- Centrifuge at 300 g for 7 min at 4°C
- Remove supernatant and place in a clean 15 ml Falcon tube
- Label falcon tubes as explained before and store at -80°C
- Resuspend protoscoleces (PSC) pellet in 1 ml PBS (or normal physiological saline), spin at 300g for 7 min at 4°C.
 - FIRST TUBE
 - Remove supernatant and discard
 - Add Paraformaldehyde 4% and resuspend gently
 - Label
 - Store vertically in fridge at 4°C for 2h 30 min
 - Remove gently the PFA (take care of not disturbing the sediment)
 - Add 5 ml of Ethanol 50% in PBS
 - Store at -80°C
 - SECOND TUBE
 - Remove supernatant and discard
 - Add 500 µl Karnowsky and resuspend gently
 - Label
 - Store at 4°C (fridge)
 - THIRD TUBE
 - Remove supernatant and discard
 - Label
 - Store at -80°C

MEMBRANES (solid parasite material)

- Collect in a sterile container
- If more than one cyst is removed, process material from different cyst separately cyst by cyst and give cyst number code on the stored material (see cyst number code on case report form)
- Store immediately at -80°C
- - Label as explained before

7. *SAMPLES WILL BE SHIPPED ONCE EVERY SIX MONTHS (OR ONCE EVERY YEAR) TO PARTNER 3.*

SHIPPING ADDRESS

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