HELIX Child panel Common SOP: Sample Collection, processing and shipping

V11. 4th July 2014

Cohorts should ensure that they are following the most up to date version of this SOP during fieldwork

Contenido

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BTHFT : Bradford Teaching Hospitals NHS Foundation Trust ; INSERM: Institut National de la Sante et de la Recherche Medicale; CREAL: Centre for Research in Environmental Epidemiology; VDU: Vytauto Didziojo Universitetas; UoC: University of Crete; CRG: Centre for Genomic Regulation; NIPH: Norwegian Institute of Public Health, Division of Environmental Medicine; ICL: Imperial College London; APA: Apa Laboratoris Clínics

Panel study design and scope of this SOP

In the Child panel study, 35 children in 5 HELIX cohorts will be monitored for one week over two periods. Over this week they will collect 2 urines samples per day (night and morning) which will be stored in the participants' freezer (until night before visit to clinic). At the end of each week they will come to clinic for examination and blood collection. At the end of the first week they will follow the normal HELIX subcohort protocol and sample processing and collection scheme. At the end of the second week they will follow a similar, but reduced protocol to the HELIX subcohort scheme (named Child Panel Reduced Clinical Follow-up).

The first part of the this SOP will detail samples collected during the first monitoring week, including urine samples collected at home and the urine and blood samples collected and processed in the same way as children in the HELIX Subcohort only.

The second part of this SOP will detail samples collected during the second monitoring week, including urine samples collected at home and urine, hair and blood samples collected and processed as part of the Reduced clinical follow-up.

Materials:

- BD Vacutainer blood collection set (1/subject, BD: 368655, pre-attached holder, butterfly clip, 23G needle).
- tube rack
- cuff
- Gloves
- disinfectant
- local anaesthetic patch (ELMA)
- gauze bandages
- medical tape
- Disposal box for needles.
- Tubes for blood collection in week 1 (1/subject, NOTE: BD tube references are the Spanish reference numbers, check number against description)
  - 6 ml EDTA vacutainers for plasma and DNA isolation (BD: 368381, dark blue top, trace element plastic, K_2EDTA coated)
  - Tempus tubes for RNA isolation (Life Technologies Cat No: 4342792, light blue top)
  - 4 ml plastic silica vacutainers for serum (BD: 368813, pink top, silica coated, clot activator)
  - 5mL glass silica vacutainer for serum (BD: 367614, red top, silica coated, no activator)
- Tubes for blood collection in week 2 (1/subject, NOTE: BD tube references are the Spanish reference numbers, check number against description)
  - 4 ml EDTA vacutainers for plasma and DNA isolation (BD: 368861, purple top, , K_2EDTA coated)
  - Tempus tubes for RNA isolation (Life Technologies Cat No: 4342792, light blue top)
  - 4 ml plastic silica vacutainers for serum (BD: 368813, pink top, silica coated, clot activator)
- Centrifuge
- Centrifuge tube : 15 ml falcon tube or 5 mL centrifuge tube (SARSTED: 55.475.001)
- Micropipette and pipette tips (HDPE, LDPE, PP, or PS plastic (recycle mark 2,4,5,and 6, check material with Cathrine Thompsen))
- For urine collection to be sent out to each family prior to subcohort visit:
  - 70 mL urine collection tubes (Sarstedt: 75.9922.744)
  - Small fridgebox (a Tupperware box that urine tubes are placed inside)
  - Cool bag
  - Ice pack
  - Printed labels for families to fill in themsleves
- 1.2 ml vials (nominal volume 1ml) external threads: (SARSTED: 72.377).
- 2.0 ml vials (nominal volume 1.8 ml) external threads (SARSTED: 72.379)
- SARSTED Coloured cap inserts: (red (8/subject), 65.386.002), yellow (14/subject, 65.386.003), green (14/subject, 65.386.004), blue (9/subject, 65.386.005), violet (8/subject).
- Plastic Boxes cryovial boxes, 9x9 samples: (SARSTED 93.877)
- Boxes for Tempus tubes (Nirco, Cat No. B29)
- Printed labels with aliquot codes as specified in this SOP
- Dry ice for eventual shipping
- For Blood smear:
  - Glass slides (26mm x76 mm 1 mm thick polished edges frosted 20 mm vacuum packed, Deltalab, box of 50 units: D100003)
  - Capillary glass tube (Sarstedt, Ref num: 51.931.100)

- Slide box for 50 slides Blue Company (Sharlab, Ref num: 027-19277A).
- Quick panoptic number 1 (Química Analítica Aplicada, Ref num: 991681)

WEEK 1: Week finishing with HELIX Subcohort follow-up

Summary of samples to be collected during the Week 1 of the child panel study:

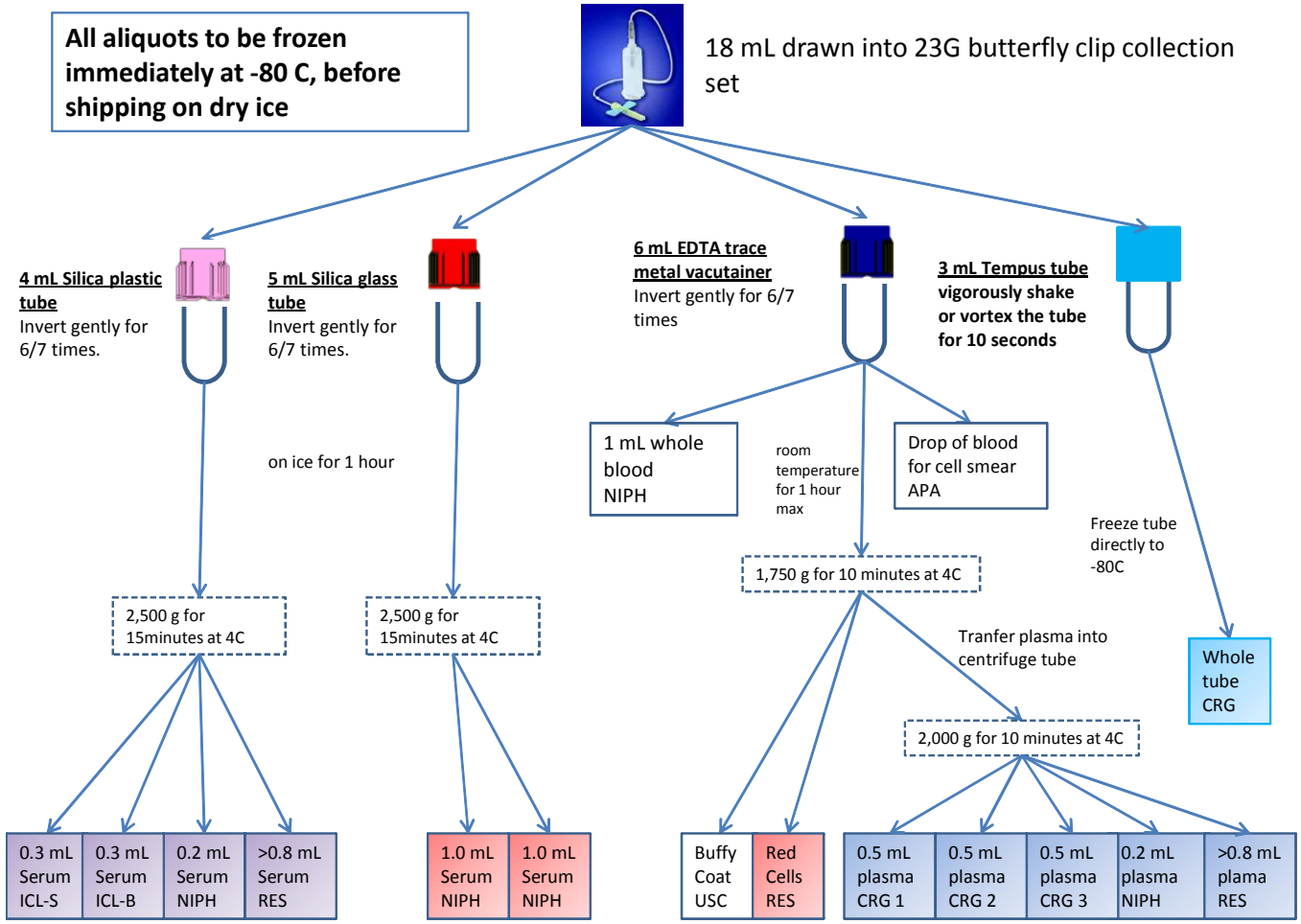
Type of sample	N*	Collection Tube	Sample processing	Sample quantity Required, mL	Purpose
Urine	35	70 mL collection containers (collected morning and evening over 8 days)	Urine	1.75	Metabonomics
				0.35	Phthalates
				0.5	Phenols
				0.5	OP Pesticides
				1.0	Metals
				0.2	Cotinine
				0.5	Creatinine, specific gravity
Blood (18 mL)	35	4 mL silica vacutainer **368813**	Serum	0.6	Metabonomics
				0.2	HDL, Cholesterol, trigycerides, glucose
				>0.8	spare
		5mL silica glass vacutainer (no additive) **367614**	Serum	2.0	PCBs, DDE, HCB, PBDE
		6 mL EDTA (trace metal tube) **368381**	Whole blood	0.9	Heavy metals
			Blood smear	0.1	Cell differentiation
			DNA	2.5	Methylomics
			Plasma	0.5	Proteomics
				1.0	miRNA
				0.2	PFASs
				>0.8	spare
		3 mL Tempus	RNA	3	Transcriptomics

* In Each Cohort

Buccal Scrapes	35^	Ependorfs pre-filled with PBS / RNA protect	Buccal Cells	10 scrapes of each cheeck	Methylomics, transcriptomics

^ In BiB, Eden, KANC and INMA cohorts only

Blood collection and processing



Procedure for blood collection:

- Blood should be collected only by trained personnel using aseptic methods.
- An EMLA plaster will be applied to child before their clinical examination at least one hour before blood collection and before blood collection following manufacturer instructions.
- Sampling location should be an isolated, peaceful area (e.g., a separate room) with all the necessary equipment prepared beforehand.
- Date and time of blood sampling should be noted on the sample data worksheet (see Annex). Food and medicines consumed that day should be specified on sample data worksheet.
- To avoid hemolysis (lysis of red blood cells) when collecting blood samples, we recommend the following procedures:
    - Follow manufacturer's instructions
    - Avoid drawing blood from a hematoma
    - Avoid frothing of the sample
    - Make sure the venipuncture site is dry
    - Avoid a probing, traumatic venipuncture
    - Avoid prolonged tourniquet application or fist clenching
    - Vacuum tubes should be filled completely

- To prevent backflow of tube additives from the tube into the individual's arm, observe the following precautions:
    - Place the individual's arm in a downward position.
    - Hold the tube with the cap up.
    - Release the tourniquet as soon as the blood starts to flow into the tube.
    - Make sure the tube contents do not touch the cap or the end of the needle during venipuncture.

- Blood will be collected into the four tubes in the following order:

    1. Plastic serum vacutainer,
    2. glass serum vacutainer
    3. EDTA,
    4. Tempus,

- The serum vacutainers and EDTA tubes should be filled completed. Blood should be drawn into the Tempus tube up to the black line on the tube.
- Immediately after collection into all tubes, The serum and EDTA tubes should be gently inverted 6-7 times. The Tempus tube should be **vigorously shaken or vortexed for 10 seconds**

Blood processing protocol

It is very important to document the time of collection of the sample, the time of the start of centrifugation, the time of freezing to -80°C. Moreover, the patient ID, the date sample taken and the type of sample should be noted in the **sample data worksheet**.

The blood will be processed in a variety of ways: Serum (silica vacutainers), Plasma and DNA (EDTA), RNA (tempus) and blood cells (future lipid metabolomics or adductomics) will be extracted. A blood smear will be made from a drop of whole blood in order to count white blood cell types.

Serum vacutainers should be put on ice or in the fridge while you proceed with processing of other samples. Tempus tubes should be put directly into the freezer.

See section on labelling samples for further information on the correct labelling.

Aliquot racks should be placed on ice while they are prepared for placing in the freezer

EDTA vacutainer: plasma and DNA

- Collect blood in 1 EDTA vacutainer (dark blue top)
- Invert gently for 6/7 times.
- Aliquot 1 mL whole blood for lead measurement into 1.8 mL cryovial
- Label this aliquot HELIXID-WB-1. Do use colour insert. Put in first column of NIPH storage box.
- Take out a drop of blood using a sterile capillary glass tube and proceed with cell count smear below.
- The remaining blood in EDTA vacutainer may be left at room temperature for a maximum of 1 hour before centrifugation
- Centrifuge the rest of the EDTA blood samples in a horizontal rotor (swing-out head) at 1,750 g for 10 minutes at 4 °C.
- Transfer the supernatant (plasma, approximately 50% of the volume) to a new pre-labelled 15 ml falcon tube. Leave a very small amount of plasma on top of the buffy coat layer, so that the buffy coat is not being disturbed and no cells are contaminating the plasma.

  Warning: Do not discard the vacutainer tube!

- Centrifuge the falcon tubes with plasma at 2,000 g for 10 minutes at 4 °C.
  Warning: Excessive centrifuge speed (over 2000 g) may cause tube breakage and exposure to blood and possible injury. If needed, RCF for a centrifuge can be calculated. For an on-line calculator tool, please refer to: http://www.changbioscience.com/cell/rcf.html

- Aliquot the plasma as follows. Be sure of not transferring any pellet residuals. If you are worried there is insufficient plasma please fill in this order: P-04 > P-03> P-02 > P-01 > P-05 (reserve):
  - 3 x 0.5 mL. BLUE colour insert. Labels: HELIXID-P1, HELIXID-P2 and HELIXID-P3. Put in first columns of separate CRG storage boxes A, B and C.
  - 0.2mL. BLUE colour insert. Label : HELIXID-P4. Put in second column of NIPH storage box.

  - Remaining plasma. (Use 1.8 mL cryovial if necessary). BLUE colour insert. Label: HELIXID- P5. Put in first column of Reserve box.
- Store the storage boxes at -80°C. Prior to shipment

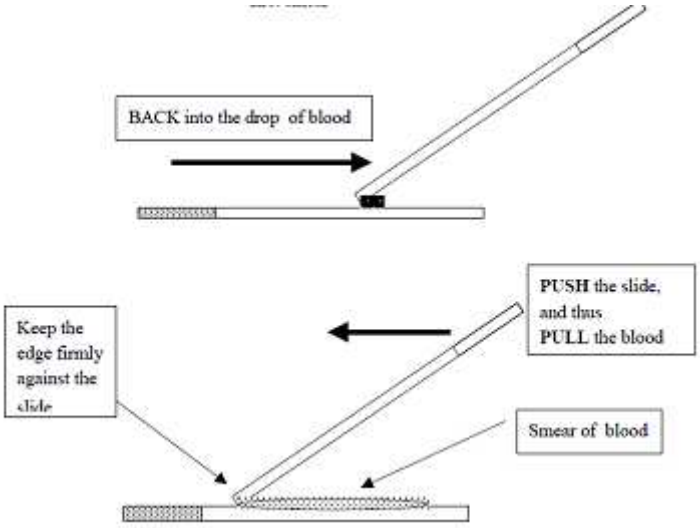
- Transfer the buffy coat (grayish layer and a small amount of the red blood cells) from the first centrifugation round, into a 1.8 mL cryovial. **Be sure you get the entire buffy coat.** The small amount of plasma left in the vacutainer may be taken as well as the buffy coat
- Label this aliquot HELIXID-BC-1. Do not use colour insert. Put in first column of USC storage box.

- Transfer the remaining contents on the vacutainer (the red blood cells) into a 1.8 mL cryovial. Label this aliquot HELIXID-RC-1. Red colour insert. Put in second column of Reserve box
- Store the storage boxes directly to -80C, prior to shipment
- Fill the sample collection document.
- **Always record exact time of collection (venipucture), centrifugation and storage to -80°C (on attached worksheet), and always record any deviation from the procedure.**

Blood smear for cell count protocol

1. Write in pencil the HELIX ID of the child on the slide matte label.
2. Perform the blood draw as shown above.
3. Invert the EDTA tube several times to homogenize the proportion of cells. Take one drop of blood from the tube using a sterile capillary glass tube.
4. Place a drop of blood approximately 4 mm in diameter on the slide (near the end).
5. Smear the blood droplet (requires training): Spread the drop by using another slide (called here the "spreader"), placing the spreader at a 45° angle and BACKING into the drop of blood. The spreader catches the drop and it spreads by capillary action along its edge. To make a short smear, hold the spreader at a steeper angle, and to make a longer smear, hold it closer to the drop. Now, push the spreader across the slide; this PULLS the blood across to make the smear. **Do not push the blood by having it ahead of the smearing slide!** It should take about one second to smear the drop. A smooth action is required, with the edge of the spreader held against the slide. This will yield a nice, even smear. Take a look at the figures and for extra information visit

http://www.youtube.com/watch?v=O3d_4dkVVSE,
http://www.youtube.com/watch?v=XBCxusLUe68&feature=endscreen&NR=1



6. Allow to dry at least 30 minutes. Be sure that the slide is completely dry!

7. Submerge the slide in the panoptic number 1 (blue, fixation solution) **for 30 seconds** and eliminate the excess of liquid by contacting the extreme of the slide with a paper.

8. Air dry until completely dried. To accelerate drying, keep the sample leaning.

9. Store it inside the slide box and leave at room temperature. They are stable for at least one month.

10. Ship slides to Apa Laboratoris Clínics every three weeks. Follow instructions for shipment in next sections.

Tempus tube: RNA

- Draw 3 mL of blood directly into each Tempus Blood RNA.
  Note: The black mark on each tube label indicates approximately 3 mL.
- Immediately after filling each Tempus tube, vigorously shake or vortex the tube for 10 seconds to ensure that the Applied Biosystems Stabilizing Reagent makes uniform contact with the sample.
  IMPORTANT! Failure to mix the stabilizing reagent with the blood leads to inadequate stabilization of the gene expression profile and the formation of microclots that can potentially compromise the RNA purification procedure.
- Label the tube HELIXID-R1. Store in Tempus tube storage box
- Store the tubes directly at -80 ºC. Prior to shipment to CRG.

IMPORTANT: Do not let the samples come into direct contact with the dry ice.

- Fill the sample collection document.
- Follow instructions for shipment in next sections.

Serum samples

- Collect blood in 1 plastic silica vacutainer (pink top) and 1 glass silica vacutainer (red top).
- Invert both gently for 6/7 times.
- Allow both to clot for **1 hour on ice.**
- Centrifuge the vacutainers at 2500g for 15 minutes, at 4^{o}C.

- Aliquot the serum from the plastic vacutainer as follows into 1 mL cryovials. If you are worried there is insufficient plasma please fill in this order:SP-02 (ICL) > SP-01 (NIPH) > SP-03 (ICL-B) > SP-04 (RES):
    - 0.2mL. VIOLET colour insert. Label : HELIXID-SP1. Put in third column of NIPH storage box.
    - 0.3mL. VIOLET colour insert. Label : HELIXID-SP2. Put in first column of ICL Sera storage box.
    - 0.3mL. VIOLET colour insert. Label : HELIXID-SP3. Put in first column of ICL_BACKUP storage box.
- Transfer remaining serum into 1.8 mL cryovials . VIOLET colour insert. Label: HELIXID-SP4. Put in third column of Reserve box

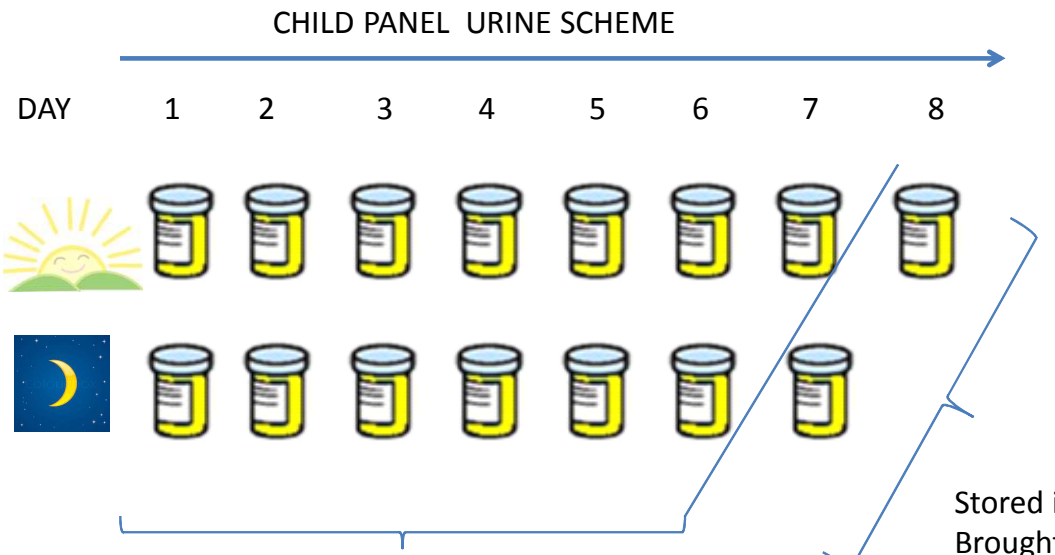
- Aliquot the serum from the glass vacutainer as follows into a 1.8 mL cryovials:
    - 1 mL. RED colour insert. Label: HELIXID-SG1. Put in fourth column of NIPH storage box
    - Remaining serum. RED colour insert. Label: HELIXID-SG2. Put in fifth column of NIPH storage box

- Store the cryovials at -80°C prior to shipment
- **Always record exact time of collection (venipucture), centrifugation and storage to -80°C (see annex 10), and always record any deviation from the procedure.**
- Keep 3 full sets of disposable equipment used for sampling and storage for blank control. Ship to NIPH together with last samples.

Technical Quality Control samples

Technical quality control samples of urine, plasma and serum will be distributed to each of the cohorts. These should be shipped 'blind' to the analysing laboratories. Further information on this process will sent on receiving the technical control samples.

Urine Collection and processing

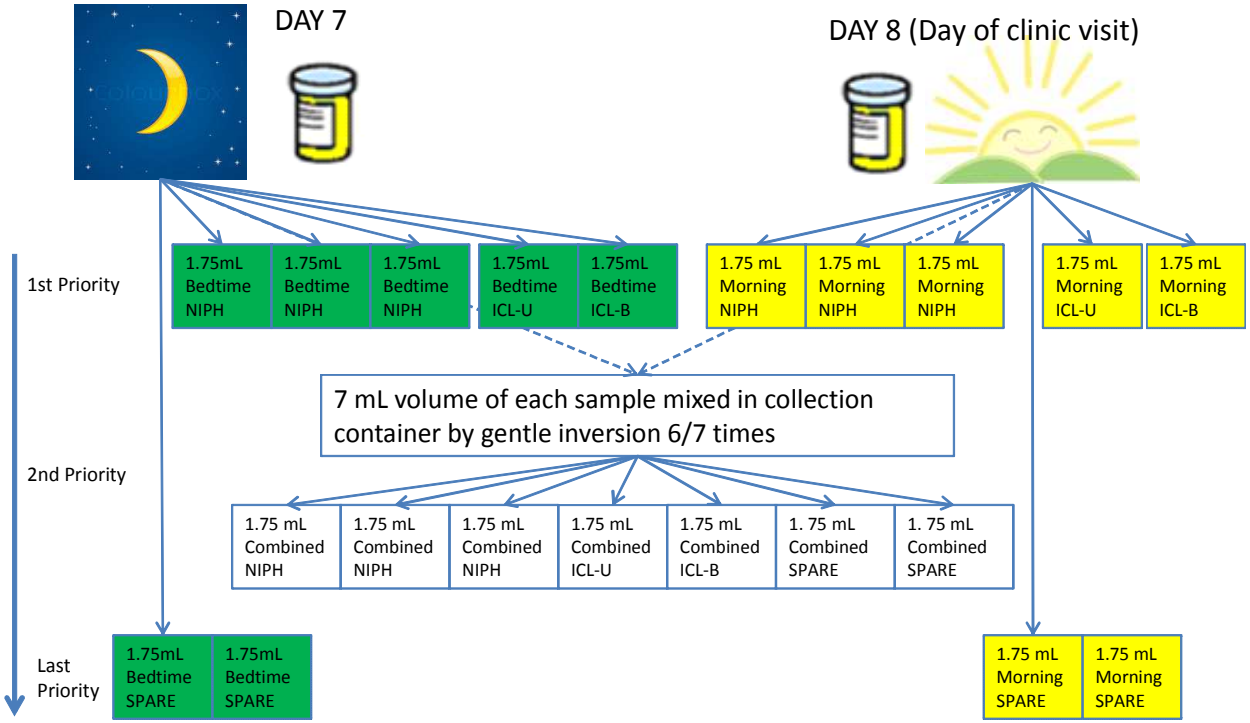


3 sets of aliquots made with following codes:

NIPH	MU1	MU2	MU3	MU4	MU5	MU6	MU7
	NU1	NU2	NU3	NU4	NU5	NU8	
ICL	MU1	MU2	MU3	MU4	MU5	MU6	MU7
	NU1	NU2	NU3	NU4	NU5	NU8	
Back-up	MU1	MU2	MU3	MU4	MU5	MU6	MU7
	NU1	NU2	NU3	NU4	NU5	NU8	

Samples nested within subcohort:
Follow subcohort aliquotting and labelling scheme: MUx, NUx, CUx

Samples collected in 70 mL containers
Samples stored at 4C after collection (>24h)
Aliquots frozen at -80C before shipping on dry ice



Urine collection

Urine samples collected from day 1 to the first morning sample of day 7 will be kept in participants freezers. Families will write the date and time of collection on each the tubes when they are collected and before placing in the freezer. If there is not space in the freezer for all the tubes they may be collected more frequently or cohorts may consider installing a temporary freezer.

On day 7, the technician will collect the frozen samples. They should not be allowed to defrost and a cool box with ice packs (or if necessary dry ice) should be used to transport the samples from home to centre without thawing. The samples should be stored frozen until they are aliquotted.

During the visit of Day 7, the technician will provide two more tubes and a cool bag and ice pack and go through instructions for the next day. These last two samples from Day 7 nightime and day 8 morning will be processed in the same way samples from the subcohort are processed.

- Stick two printed labels (See example) on two collection tubes. Place tubes into fridgebox. Place fridgebox into cool bag alongside ice pack. This will be sent to families as part of information pack prior to visit.
- Parents will be instructed how to fill and store the samples (see information for families).

Subject ID: 123

Date: _ _ / _ _ / 20 _ _

Time of urination: _ _ : _ _ hours

Infancia y Medio Ambiente

On arrival at study centre:

- Parents will bring two samples with them to centre (inside their cool bag), which will be placed in 4^{o}C fridge on arrival. Check labels are completed correctly (ask mother for to help fill in if it is not completed).
- If the family has not brought samples, ask child to provide new urine sample in collection tube when given opportunity to go to toilet (this sample should be labelled as CUx samples and a note made on comment section of sample data sheet).

Urine sample processing

Samples from Day 1 morning to Day 7 morning:

The frozen samples will be allowed to defrost overnight in a fridge (4 ^{o}C). We recommend doing this the night before day 8 so all of the samples from each child can be processed together. Take out of fridge 30 minutes before aliquotting to bring to room temperature. The samples should be inverted gently 2-3 times before aliquotting.

From each sample make three aliquots of 1.75 mL in a 2 mL cryovial, with no colour insert:

The samples should be labeled with aliquot code (including HELIX ID) with a number corresponding to the day of collection and if it is a morning or night urine

- Day 1 morning: MU1
- Day 1 night: NU1

- Day 2 morning: MU2
- Day 2 night: NU2
- ….
- Day 6 morning: MU6
- Day 6 night: NU6
- Day 7 morning: MU7

Put each of the aliquots in the following boxes:

- Aliquot 01 in NIPH storage box (in order of collection immediately following the blood samples)
- Aliquot 02 in ICL urine storage box (in order of collection)
- Aliquot 03 in Reserve box (in order of collection immediately following the blood samples)

Samples from Day 7 Nighttime and Day 8 morning (following HELIX subcohort processing)

- The samples that children have bought with them that day will be collected from fridge.
- Aliquot in the order specified below.
- Aliquot the urine from the 'bedtime sample' tube as follows into 2 mL cryovials:
    - 3 x 1.75 mL. GREEN colour insert. Label HELIXID- NUx-01, HELIXID-NUx-02 and HELIXID-NUx-03. Put in NIPH storage box immediately following the urine samples collected over the week.
    - 1 x 1.75 mL. Green colour insert. Label HELIXID- NUx-04. Put in ICL urine storage box immediately following the urine samples collected over the week.
    - 1 x 1.75 mL. Green colour insert. Label HELIXID- NUx-05. Put in second column of ICL_BACKUP storage box
- Aliquot the urine from the 'morning sample' tube as follows into 2 mL cryovials:
    - 3 x 1.75 mL. YELLOW colour insert. Label HELIXID- MUx-01, HELIXID- MUx-02 and HELIXID-MUx-03. Put in NIPH storage box immediately following the NUx urine aliquots
    - 1 x 1.75 mL. YELLOW colour insert. Label HELIXID- MUx-04 . Put in ICL urine storage box immediately following the NUx urine aliquots
    - 1 x 1.75 mL. YELLOW colour insert. Label HELIXID- MUx-05. Put in third column of ICL_BACKUP storage box
- Add 7 mL 'First morning sample' and 7mL 'Bed time sample' to a pp collection container. Invert the container 6 or 7 times to mix.
- From this mixture aliquot out the following into 2 mL cryovials:
    - 3 x 1.75 mL. Do not use colour insert. Label HELIXID- CUx-01, HELIXID-CUx-02, HELIXID-CUx-03. Put in NIPH storage box immediately following the MUx urine aliquots
    - 1 x 1.75 mL. Do not use colour insert. Label HELIXID- CUx-04. Put in ICL urine storage box immediately following the MUx urine aliquots

  - 1 x 1.75 mL. Do not use colour insert. Label HELIXID- CUx-05. Put in fourth column of ICL_BACKUP storage box
  - 2 x 1.75 mL. Do not use colour insert. Label HELIXID- CUx-06 and HELIXID-CUx-07. Put in RESERVE storage box immediately following the MUx urine aliquots (after they have been prepared in next steps)
- Then, if urine left in the 'first morning sample' and 'bed time sample', do following aliquots:
  - 2 x 1.75 mL 'bed time sample'. GREEN colour insert. Label HELIXID- NUx-06 and HELIXID-NUx-07. Put in RESERVE storage box immediately following the urine samples collected over the week.
  - 2 x 1.75 mL. 'mornisng sample'. YELLOW colour insert. Label HELIXID- MUx-06 and HELIXID-MUx-07. Put in RESERVE storage box immediately following the NUx urine aliquots
- Freeze the cryovials at -80°C. Prior to shipment.
- **Always record exact time of urine collection and storage to -80 ºC (on attached worksheet), and always record any deviation from the procedure.**

Blank Control (for urine samples)

Keep 3 full sets of disposable equipment (pipette tips, 1mL and 1.8mL cryovials, 70 mL collection tubes) used for sampling and storage for blank control whenever the batch is changed. Note the HELIX-IDs of sample using that batch of equipment. Ship to NIPH together with the last samples.

Cohort Quality control (for urine samples)

On collection of the urine from **the last child** in each season of the panel study a 'quality control' sample should be made. Spare urine (not required for aliquotting as detailed above) should be combined to half fill a 70 mL collection container.

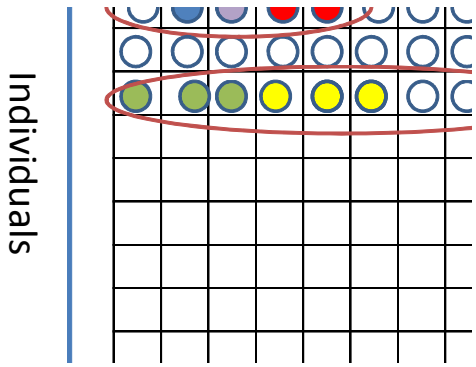
From this 10 x 1.75 mL aliquots should be made (2mL cryovial, no colour insert). They should labelled with the cohort code and placed in each ICL urine box on the bottom row.

Storage of samples from panel study week 1:

Each centre should have the following box ready in their freezers to be filled as follows. They should be number consecutively as they are filled to keep track of samples within each box (eg NIPH Storage Box 1, NIPH storage box 2 etc..)

NIPH STORAGE BOX

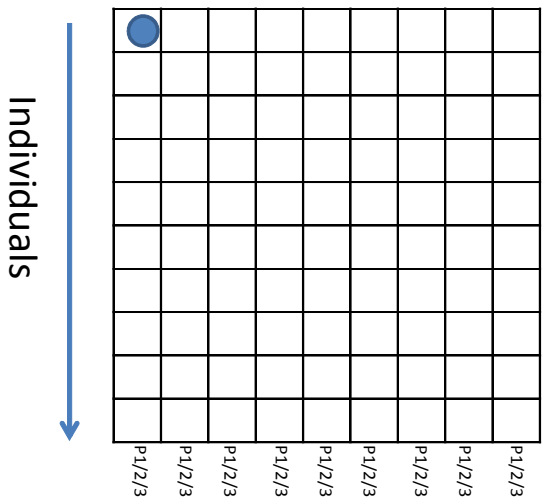
Blood samples:
WB-01; P-04; SP-01; SG-01, SG-02



Day 1 – day 7 morning samples with numbered by day aliquot codes

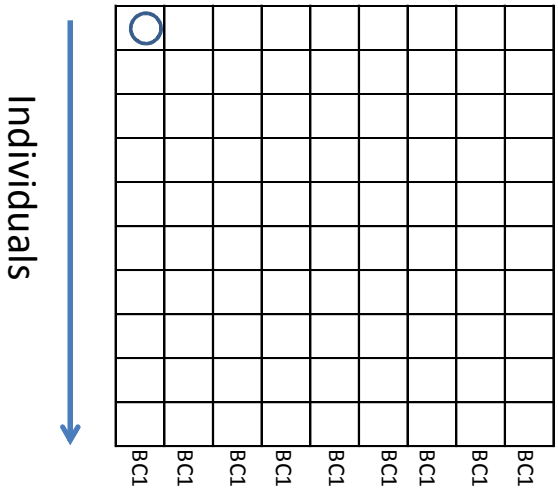
Day 7 night and Day 8 morning samples with subcohort aliquot codes: NUx, MUx, CUx

CRG STORAGE BOX A/B/C



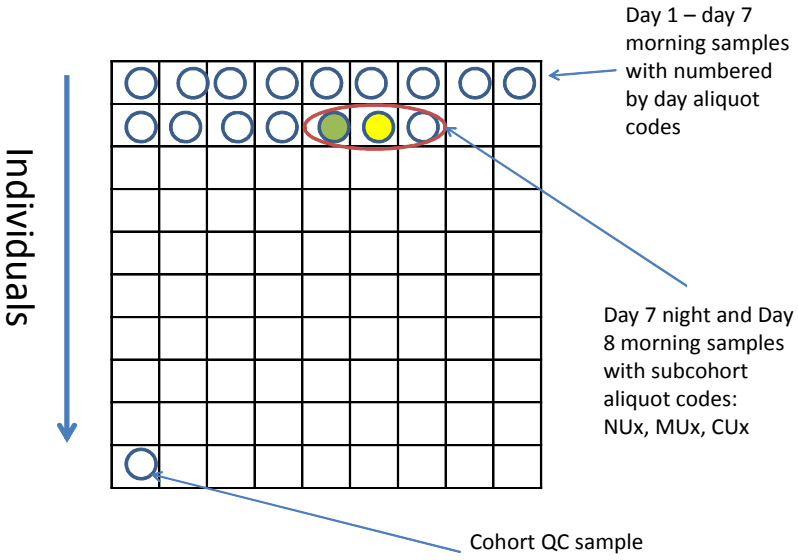
NB: Filled down rows first with consecutive individuals , before new column started. Same scheme followed for CRG storage boxes A, B and C

USC STORAGE BOX

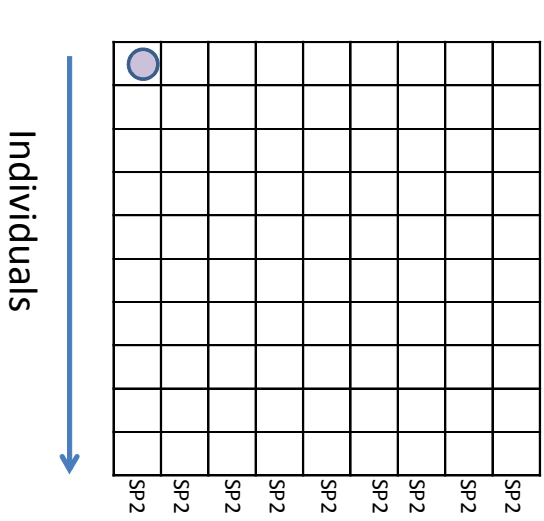


NB: Filled down rows first with consecutive individuals , before new collumn started

ICL URINE STORAGE BOX

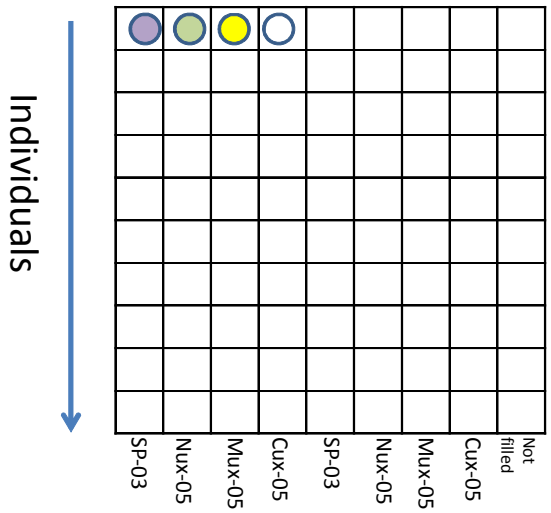


ICL SERA STORAGE BOX



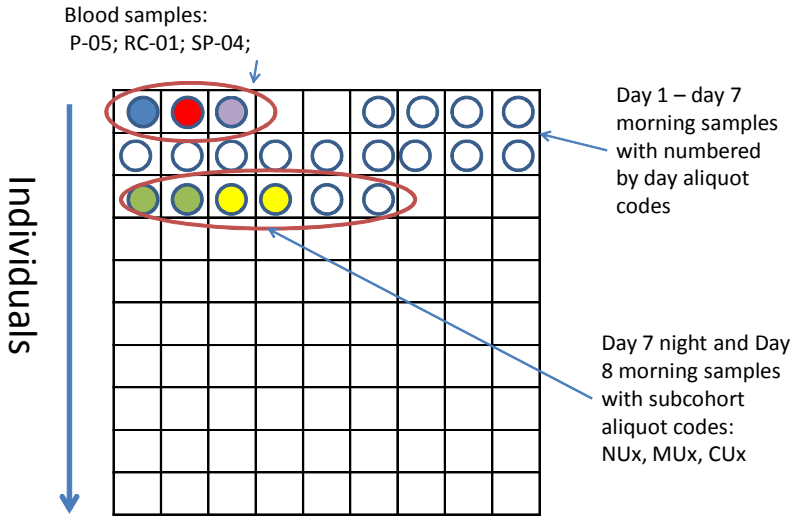
NB: Filled down rows first with consecutive individuals , before new collumn started

ICL_BACKUP STORAGE BOX



NB: Filled down rows first with consecutive individuals , before new collumn started

RESERVE STORAGE BOX



In addition there should be a:

- TEMPUS tube box (stored at -80C until shipment to CRG)
- Cell Smear Slide Box (Stored at Room temperature before shipment to **Apa Laboratoris Clínics** every three weeks)

WEEK 2: Week finishing with Reduced Clinical follow-up

Summary of samples to be collected during the week 2 of Child panel studypanel:

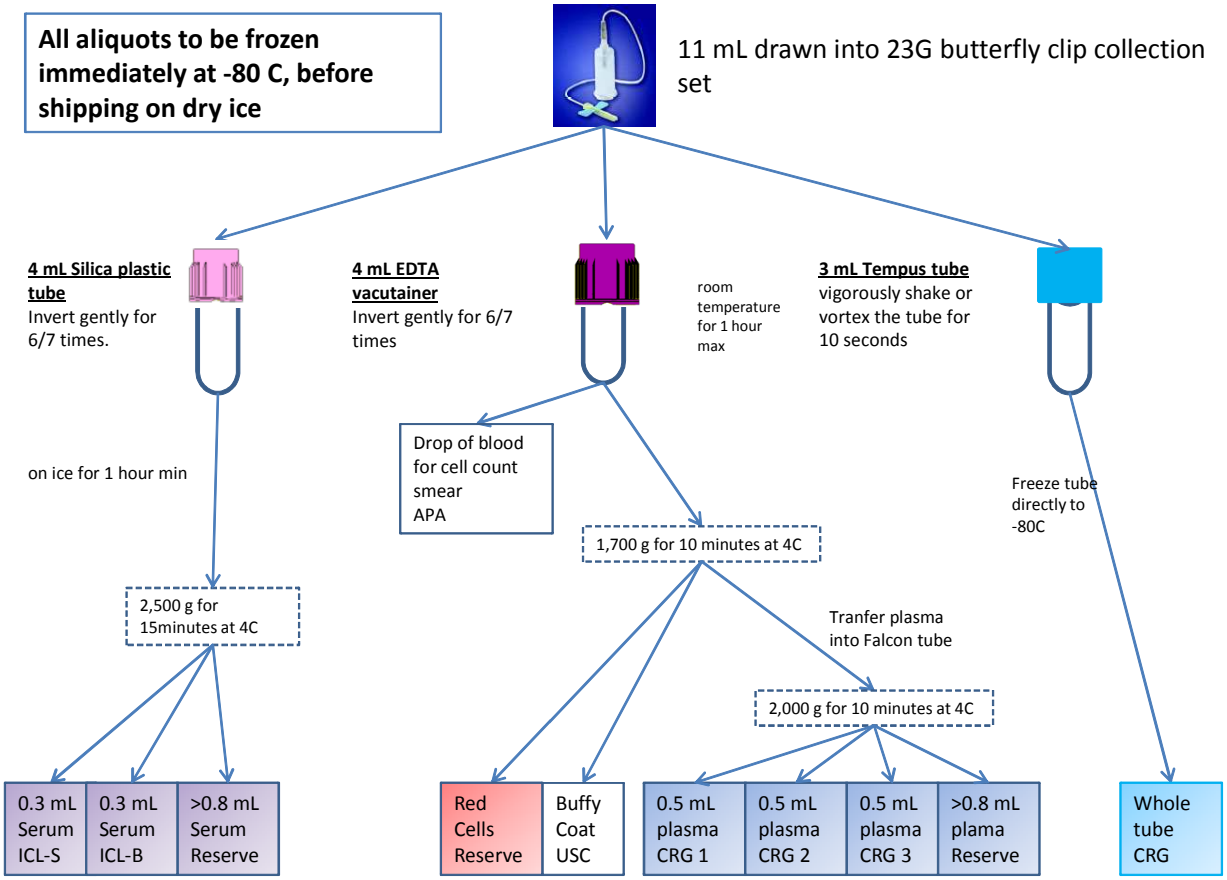
Type of sample	N *	Tube	Sample processing	Sample quantity Required, mL	Purpose
Urine	150	70 mL collection containers (collected morning and evening over 8 days)	Urine	1,75	Metabonomics
				0.35	Phthalates
				0.5	Phenols
				0.5	OP Pesticides
				0.5	Creatinine, specific gravity
Blood	150	4 mL silica vacutainer **368813**	Serum	0.6	Metabonomics
				0.2	HDL, Cholesterol, trigycerides, glucose
				>0.8	spare
		4 mL EDTA (**368861)**	Blood smear	0.1	Cell count
			DNA	2	Methylomics
			Plasma	0.5	Proteomics
				1.0	miRNA
				>0.4	spare
		3 mL Tempus	RNA	3	Transcriptomics
Hair	150	Zip-lock bag	Hair	20 mg	Nicotine /Cotinine

* From each cohorts

Buccal Scrapes	35^	Ependorfs pre-filled with PBS / RNA protect	Buccal Cells	10 scrapes of each cheeck	Methylomics, transcriptomics

^From Eden, BiB and INMA cohorts only

Blood collection and processing



Blood collection

- Blood should be collected only by trained personnel using aseptic methods.
- An EMLA plaster will be applied to child before their clinical examination at least one hour before blood collection and before blood collection following manufacturer instructions.
- Sampling location should be an isolated, peaceful area (e.g., a separate room) with all the necessary equipment prepared beforehand.
- Date and time of blood sampling should be noted on the sample data worksheet (see annex). Food and medicines consumed that day should be specified on sample data worksheet.
- To avoid hemolysis (lysis of red blood cells) when collecting blood samples, we recommend the following procedures:
    - Follow manufacturer's instructions
    - Avoid drawing blood from a hematoma
    - Avoid frothing of the sample
    - Make sure the venipuncture site is dry
    - Avoid a probing, traumatic venipuncture
    - Avoid prolonged tourniquet application or fist clenching
    - Vacuum tubes should be filled completely

- To prevent backflow of tube additives from the tube into the individual's arm, observe the following precautions:
    - Place the individual's arm in a downward position.
    - Hold the tube with the cap up.
    - Release the tourniquet as soon as the blood starts to flow into the tube.
    - Make sure the tube contents do not touch the cap or the end of the needle during venipuncture.

- Blood will be collected into the three tubes in the following order:

    1. Plastic serum vacutainer,
    2. EDTA,
    3. Tempus,

- The serum vacutainers and EDTA tubes should be filled completed. Blood should be drawn into the Tempus tube up to the black line on the tube.

- Immediately after collection into all tubes, The serum and EDTA tubes should be gently inverted 6-7 times. The Tempus tube should be **vigorously shaken or vortexed for 10 seconds**

Blood processing protocol

It is very important to document the time of collection of the sample, the time of the start of centrifugation, the time of freezing to -80°C. Moreover, the patient ID, the date sample taken and the type of sample should be noted in the **sample data worksheet**.

Serum vacutainers should be put on ice or in the fridge while you proceed with processing of other samples. Tempus tubes should be put directly into the freezer.

See section on labelling samples for further information on the correct labelling.

Aliquot racks should be placed on ice while they are prepared for placing in the freezer

EDTA vacutainer: plasma and DNA

- Collect blood in 1 EDTA vacutainer (dark blue top)
- Invert gently for 6/7 times.
- Take out a drop of blood using a sterile capillary glass tube and proceed with cell count smear below.
- The remaining blood in EDTA vacutainer may be left at room temperature for a maximum of 1 hour before centrifugation
- Centrifuge the rest of the EDTA blood samples in a horizontal rotor (swing-out head) at 1,700 g for 10 minutes at 4°C.
- Transfer the supernatant (plasma, approximately 50% of the volume) to a new pre-labelled 15 ml falcon tube. Leave a very small amount of plasma on top of the buffy coat layer, so that the buffy coat is not being disturbed and no cells are contaminating the plasma.

  Warning: Do not discard the vacutainer tube!

- Centrifuge the falcon tubes with plasma at 2,000 g for 10 minutes at 4°C.
  Warning: Excessive centrifuge speed (over 2000 g) may cause tube breakage and exposure to blood and possible injury. If needed, RCF for a centrifuge can be calculated. For an on-line calculator tool, please refer to: http://www.changbioscience.com/cell/rcf.html

- Aliquot the plasma as follows. Be sure of not transferring any pellet residuals.
  - 3 x 0.5 mL. BLUE colour insert. Labels: HELIXID-P1, HELIXID-P2 and HELIXID-P3. Put in first columns on CRG storage boxes A, B and C.
  - Remaining plasma. (Use 1.8 mL cryovial if necessary). BLUE colour insert. Label: HELIXID- P4. Put in first column of Reserve box.
- Store the storage boxes at -80°C. Prior to shipment

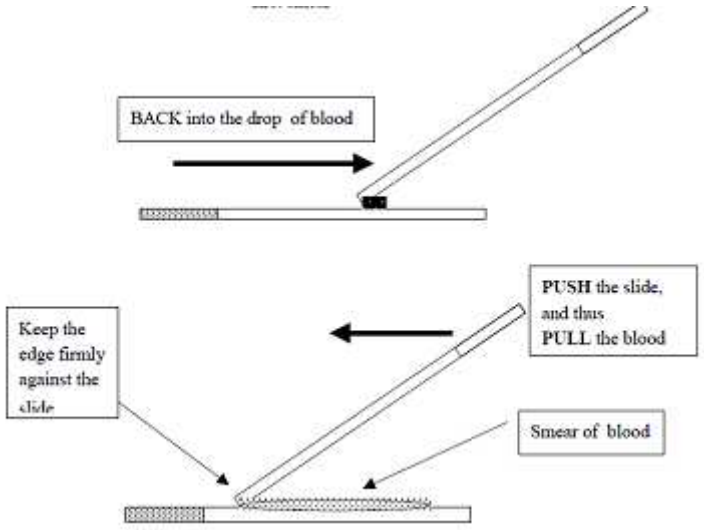
- Transfer the buffy coat (grayish layer and a small amount of the red blood cells) from the first centrifugation round, into a 1 mL cryovial. **Be sure you get the entire buffy coat.** The small amount of plasma left in the vacutainer may be taken as well as the buffy coat
- Label this aliquot HELIXID-BC-1. Do not use colour insert. Put in first column of USC storage box.

- Transfer the remaining contents on the vacutainer (the red blood cells) into a 1.8 mL cryovial. Label this aliquot HELIXID-RC-1. Use a RED colour insert. Put in second column of Reserve box
- Store the storage boxes directly to -80C, prior to shipment
- Fill the sample collection document.
- **Always record exact time of collection (venipucture), centrifugation and storage to -80°C (on attached worksheet), and always record any deviation from the procedure.**

Blood smear for cell count protocol

1. Write in pencil the id of the child on the slide matte label.
2. Perform the blood draw as shown above.
3. Invert the EDTA tube several times to homogenize the proportion of cells. Take one drop of blood from the tube using a sterile capillary glass tube.
4. Place a drop of blood approximately 4 mm in diameter on the slide (near the end).
5. Smear the blood droplet (requires training): Spread the drop by using another slide (called here the "spreader"), placing the spreader at a 45° angle and BACKING into the drop of blood. The spreader catches the drop and it spreads by capillary action along its edge. To make a short smear, hold the spreader at a steeper angle, and to make a longer smear, hold it closer to the drop. Now, push the spreader across the slide; this PULLS the blood across to make the smear. Do not push the blood by having it ahead of the smearing slide! It should take about one second to smear the drop. A smooth action is required, with the edge of the spreader held against the slide. This will yield a nice, even smear. Take a look at the figures and for extra information visit

http://www.youtube.com/watch?v=O3d_4dkVVSE,
http://www.youtube.com/watch?v=XBCxusLUe68&feature=endscreen&NR=1



6. Allow to dry at least 30 minutes. Be sure that the slide is completely dry!

7. Submerge the slide in the panoptic number 1 (blue, fixation solution) **for 30 seconds** and eliminate the excess of liquid by contacting the extreme of the slide with a paper.

8. Air dry until completely dried. To accelerate drying, keep the sample leaning.

9. Store it inside the slide box and leave at room temperature. They are stable for at least one month.

10. Ship slides to Apa Laboratoris Clínics every three weeks. Follow instructions for shipment in next sections.

Tempus tube: RNA

- Draw 3 mL of blood directly into each Tempus Blood RNA.
  Note: The black mark on each tube label indicates approximately 3 mL.
- Immediately after filling each Tempus tube, vigorously shake or vortex the tube for 10 seconds to ensure that the Applied Biosystems Stabilizing Reagent makes uniform contact with the sample.
  IMPORTANT! Failure to mix the stabilizing reagent with the blood leads to inadequate stabilization of the gene expression profile and the formation of microclots that can potentially compromise the RNA purification procedure.
- Label the tube HELIXID-R1. Store in Tempus tube storage box
- Store the tubes directly at -80 ºC. Prior to shipment to CRG

IMPORTANT: Do not let the samples come into direct contact with the dry ice.

- Fill the sample collection document.
- Follow instructions for shipment in next sections.

Serum samples

- Collect blood in 1 plastic silica vacutainer (pink top).
- Invert gently for 6/7 times.
- Allow to clot for **1 hour on ice.**
- Centrifuge the vacutainers at 2500g for 15 minutes, at 4°C.

- Aliquot the serum from the plastic vacutainer as follows into 1 mL cryovials:
  - 0.3mL. VIOLET colour insert. Label : HELIXID-SP1. Put in first column of ICL sera storage box.
  - 0.3mL. VIOLET colour insert. Label : HELIXID-SP2. Put in first column of ICL_BACKUP storage box.
- Transfer remaining serum into 1.8 mL cryovials . VIOLET colour insert. Label: HELIXID-SP3. Put in third column of Reserve box

- Store the cryovials at -80°C prior to shipment
- **Always record exact time of collection (venipucture), centrifugation and storage to -80°C (see annex 10), and always record any deviation from the procedure.**
  Keep 3 full sets of disposable equipment used for sampling and storage for blank control. Ship to NIPH together with last sample

Urine Collection and processing

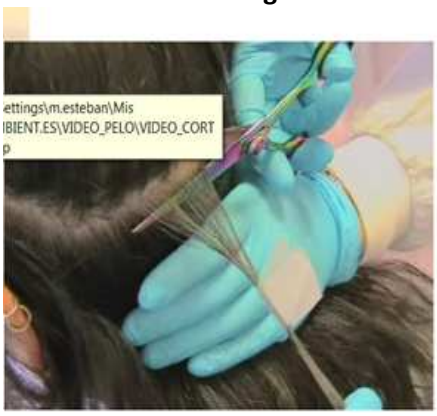
Urine collection and processing in week 2 is exactly the same as in week 1.

Hair collection protocol

Materials:

- Scissors
- Adhesive tape
- polyethylene bag, zip seal type
- Marker Pen

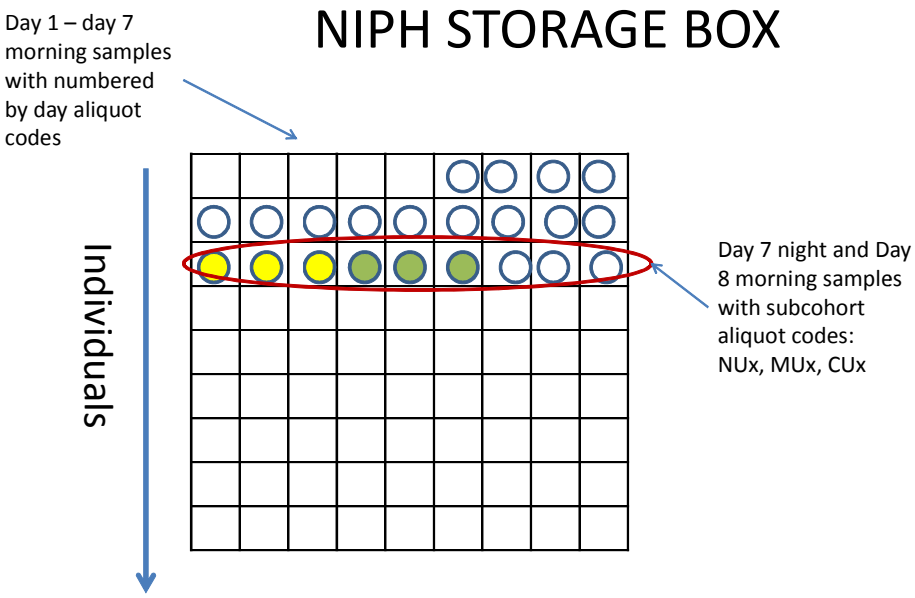
• Collect a lock of hair (about *20 mg).* Try to cut as close as possible to the root and in the occipital area of the head (the back). If longer that 5 cm an adhesive tape should be put on to mark the root. **Training will be needed for this procedure.**

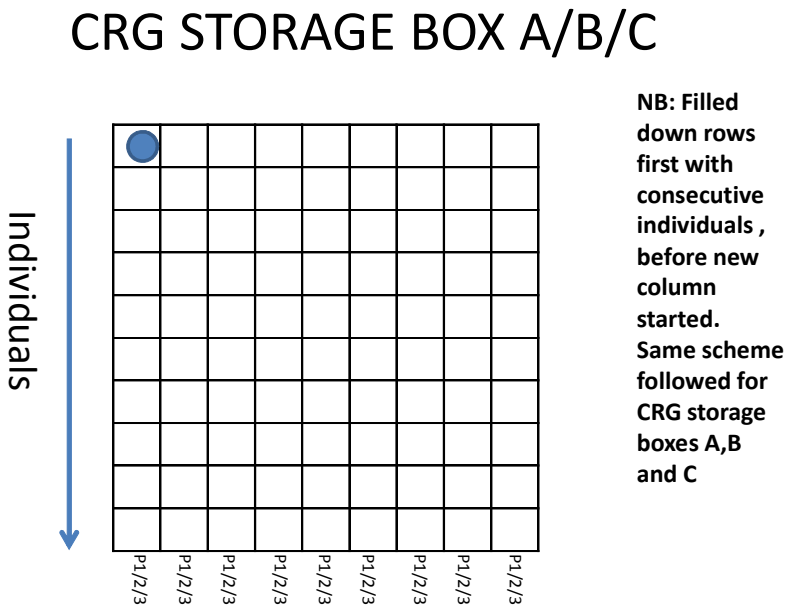


• Save all the lock cut. In the event that the child has short hair is cut in several places so that it can make up to the volume of about 20 mg.
• It is stored in a polyethylene bag zip seal type.
• Identified with a label with the date and HELIX ID NUMBER
• It will keep at room temperature in a sealed bag to avoid contamination.
- ***Ship to CREAL when all samples collected***

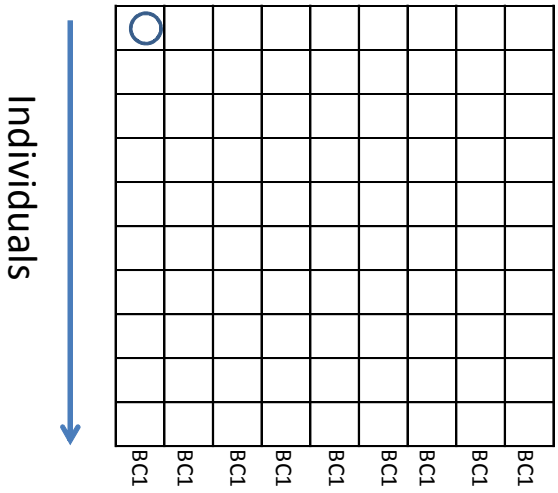
Storage of samples from panel study week 2:

Each centre should have the following box ready in their freezers to be filled as follows. They should be number consecutively as they are filled to keep track of samples within each box (eg NIPH Storage Box 1, NIPH storage box 2 etc..)



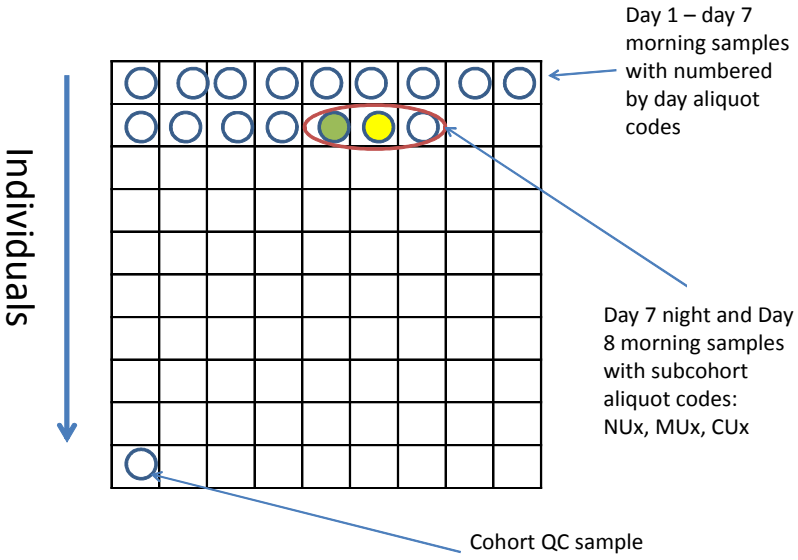


USC STORAGE BOX

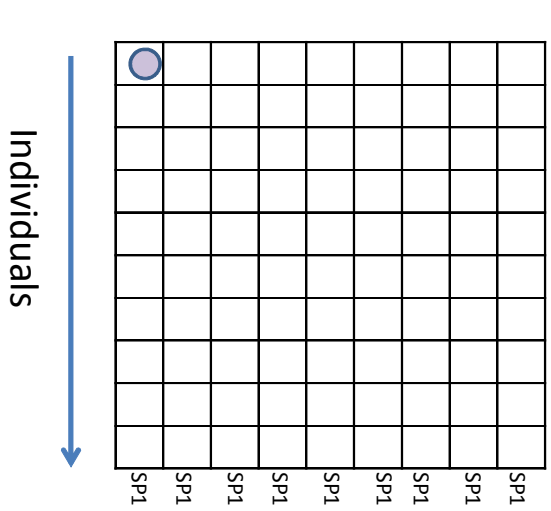


NB: Filled down rows first with consecutive individuals , before new collumn started

ICL URINE STORAGE BOX



ICL SERA STORAGE BOX



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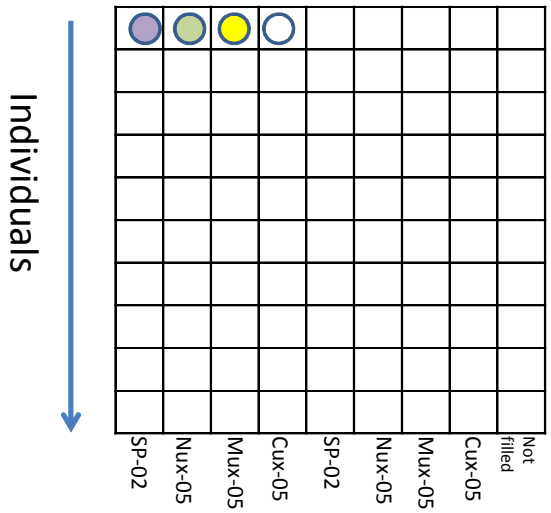
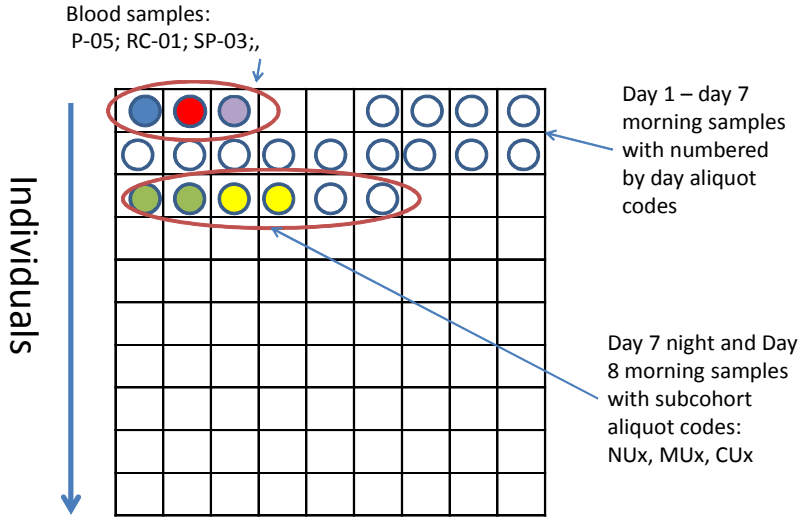
ICL_BACKUP STORAGE BOX



NB: Filled down rows first with consecutive individuals , before new collumn started

RESERVE STORAGE BOX



In addition there should be a:

- TEMPUS tube box (stored at -80C until shipment to CRG)
- Cell Smear Slide Box (Stored at Room temperature before shipment to **Apa Laboratoris Clínics** every three weeks)
- Box for Hair (shipped to CREAL).

Labelling of samples

All aliquots should be labelled with pre-printed labels containing just the aliquot code.

The aliquot code will be made up as follows

Cohort code _ Existing Cohort specific ID Number _Study code_ (these three parts will make up the HELIX subject ID) _aliquot type_aliquot number

Note samples collected from children in the first week of the panel study will have study code 1a. Samples collected from children in the second week of the panel study will have study code 1b.

Aliquot Coding Key:

HELIX ID Number			**Aliquot specific code**	
Cohort Code	Existing Cohort specific ID Number	Study code	Sample type	Aliquot number
SAB= INMA -Sabadel	xxxx	0 = Mother (archived)	MU1= Morning urine from day 1	01
EDP= EDEN - Poitiers	xxxx	1x = Child participating in subcohort only	NU1= Bedtime urine from day 1	02
KAN = KANC	xxxx	1a= Child (finishing first period of panel study)	MU6= Morning urine from day 6	03
RHE= RHEA	xxxx	1b= Child (finishing second period of panel study)	.... MU7 =Morning urine from day 7	...
BIB= BiB		2a= Pregnant women first period panel study	**NUx= Bedtime urine from day 7**	
MOB = MoBa		2b= Pregnant women second period panel study	**MUx= Morning urine from day 8**	
OSL = Oslo			**CUx= Combined urine from bedtime urine from day 7 and morning urine from day 8**	

BCN = Barcelona			BC=Buffy coat	
GRE = Grenoble			WB=Whole Blood	
			P=Plasma	
			R=RNA (tempus tube)	
			SP=Serum (from plastic tube)	
			SG=Serum (from glass tube)	
			RC=Red blood cells (from EDTA tube)	

Example:

SAB-223-1a-NUx-01 indicates the **first aliquot** made of the **bedtime urine** sample from **child 223** collected at the **first week of the child panel study** in the **INMA – Sabadell** cohort.

Coding of urine samples is a little complex to reflect the differences between samples collected over the week and those samples collected in the same way as urine samples collected as part of the 'subcohort follow-up'

Samples have sample codes reflecting if it is a morning or nighttime sample and which day it is collected on:

Day 1 morning: MU1

Day 1 night: NU1

Day 2 morning: MU2

....

Day 6 morning: MU6

Day 6 night: NU6

Day 7 morning MU7

Until the night sample of day 7 and the morning sample of day 8 where they have the following codes to reflect equivalence to the subcohort samples:

Day 7 evening: NUx

Day 8 morning: MUx

There is also a code for these urines combined: CUx

Further examples are as follows:

INMA-Sabadell ID 030, panel 1st period, morning urine Day 3, 3rd aliquot --> Sab_030_1a_MU3_03

INMA-Sabadell ID 030, panel 1st period, night urine Day 5, 3rd aliquot --> Sab_030_1a_NU5_03

Sabadell ID 030, panel second period, night urine Day 7, 4th aliquot --> Sab_030_1b_NUx_04 (this code to integrate with the subcohort labelling)

Sabadell ID 030, panel second period, morning urine Day 8, 4th aliquot --> Sab_030_1b_MUx_04

Sabadell ID 030, panel second period, combined urine, 4th aliquot --> Sab_030_1b_CUx_04

Sabadell ID 030, panel first period, combined urine, 4th aliquot --> Sab_030_1a_CUx_04

Tracking and shipping of samples

Central database

As samples are collected, aliquot information should be entered onto the sample data form. This data can be entered immediately or at a later date into the HELIX questionnaire application.

Additionally the 'Sample tracking Excel' should be completed. This should be completed and uploaded periodically onto the HELIX website (WP1). The Excel should be saved as 'Sample tracking_Child panel 1 (*or 2)_Cohort code_date uploaded* '

Shipping

ALWAYS give notice of shipment date before sending samples and let laboratories know the tracking number. Send the receiving laboratories the 'Sample tracking Excel' listing all the boxes contained in that shipment. Please copy in Oliver Robinson (orobinson@creal.cat)

Samples should be shipped on a Monday, or Tuesday at the latest

Blood and urine samples should be shipped according to the instructions below, frozen with **sufficient dry ice** to prevent thawing

- NIPH (NIPH Storage box) when enough samples are collected for suitable shipment
- For the following labs please send available samples at the end of these months: June 2014 ;October 2014; December 2014; March 2015; June 2015
    - CRG epigenetic laboratory (CRG Storage Box A, CRG Storage Box B and Tempus Tubes, sent in separate package). CRG A and Tempus can be sent in the same package
    - CRG proteomics laboratory (CRG storage box C sent separately).
    - ICL (ICL SERA and URINE STORAGE Boxes)
    - USC (USC storage box) on completion

Blood smear slides should be **sent every month** to **Apa Laboratoris Clínics.** The boxes for the blood smears will be returned to CREAL for eventual redistribution to cohorts. Send an email to Roser Clavell (roser.clavell@apalaboratoris.com) informing about the shipment (add Mariona Bustamante in copy: mariona.bustamante@crg.es). Attach to the mail an exel file named "HELIX_[cohort]_APA_shipment#_[date(20140310)]" that contains the list of IDs that are sent (where [cohort] is cohort name, # is consecutive shipment number and [date] is date of shipment).

Hair samples should be sent on completion of study to **CREAL**

Cohorts should keep the Reserve and ICL-backup boxes in their respective cohorts until required by the HELIX project.

Packaging of samples

According to the **WHO/HSE/GCR/2012.12** "*Guidance on regulations for the Transport of Infectious Substances 2011-2012"* the sample materials are to be considered as infectious substances of category B. This implies the packaging and shipment of samples to follow the "Basic triple packaging system" as described below:

Primary receptacle:

All samples should be placed in specified cryovials sealed by water-proof screw caps. All cryovials should further be placed in a storage box with a capacity of 81 tubes.
The storage boxes containing the cryovials are to be considered as primary receptacles.

Secondary packaging:

The storage boxes must be enclosed by a secondary durable and watertight packaging. Between the first and second layer of packaging there should be enough **absorbent material** to ensure that all liquid sample material will be absorbed in case of brakeage or leakage.

Outer packaging:

The samples should further be packed in a sturdy outer packing of Styrofoam boxes of sufficient size to give room for enough dry ice to keep samples cooled/frozen during transport, as well as to give room to enough cushioned material to insulate samples from physical damages. They should preferably have think walls (eg 6 cm) to prevent thawing during shipping (that may take up to a week for South to North Europe)

Dry ice:

Plenty of dry ice should be used to prevent thawing.

Documentation:

Outside outer packaging should be a Proforma invoice (low value) and a print of the Waybill that has been filled in online (full description). Both Proforma invoice and the Waybill should have "UN3373, BIOLOGICAL SUBSTANCE, CATEGORY B, and UN1845 dry ice"!) written on it, (a letter from the university/inst explaining that the samples will be used for research only, to speed up Customs.

A separate document inside the box should list all the codes of aliquots contained in package to aid receiving laboratories

Marking of the outer packaging

1. Name and address of the sender of the samples. This *must* also include phone numbers, e-mail addresses and name of contact person with knowledge of the shipment.

2. Name and address of the receiver of the samples. This should also include phone numbers, e-mail addresses and name of contact person.

3. A sticker containing the UN 3373-symbol (see below) as well as the proper shipping name (PSN) "BIOLOGICAL SUBSTANCE, CATEGORY B" next to the sticker.

BIOLOGICAL SUBSTANCE, CATEGORY B

4: Since the package also includes dry ice and will be transported by aircraft a sticker containing the “UN-1845 Carbon Dioxide, solid (dry ice)”-symbol, as well as the UN1845-sticker containing information on the amount of dry ice in the package.

5: All irrelevant labels and marks should be removed

Shipping Address and Contacts:

ALWAYS give notice of shipment date before sending samples and let laboratories know the tracking/ Airway Bill numbers numbers. WorldCourier is the recommended courier service.

NIPH:

Dr Cathrine Thomsen
Norwegian Institute of Public Health
Div. of Environmental Medicine
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0456 Oslo, Norway

Att: Cathrine Thomsen
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CREAL
Dr Oliver Robinson
CREAL
Av Dr. Aiguader 88
08003 Barcelona, Spain

Sample Data Sheet (Child Panel Week 1)

Fieldworker: _____

Day of week: _____

Date: | | | | | 2 | 0 | | |

(day) (month) (year)

Helix ID: _____

Blood sampling

Vacutainers collected (Tick all that apply):

- ☐ EDTA,
- ☐ Tempus,
- ☐ Plastic serum vacutainer,
- ☐ glass serum vacutainer

Action	Time performed (24 hours)	
Blood sampling (venopunture)		
Start of Centrifugation of EDTA tube		
Freezing of aliquots from EDTA tube to -80°C		
Freezing Tempus tube to -80°C		
Start of centrifugation of plastic serum tube		
Start of centrifugation of glass serum tube		
Time of freezing of serum aliquots to -80°C		

Blood Aliquots made (Tick all that apply):

- ☐ WB-01
- ☐ Blood Smear
- ☐ P-01
- ☐ P-02
- ☐ P-03
- ☐ P-04
- ☐ P-05
- ☐ BC-01
- ☐ RC-01
- ☐ SP-01
- ☐ SP-02
- ☐ SP-03
- ☐ SP-04
- ☐ SG-01
- ☐ SG-02

Comments on blood processing (Eg. Any deviation from protocol)

_____
_____
_____

Weekly Urine samples (Day 1 – Day 7 morning)

Sample (tick all collected)	Aliquots (tick all obtained)		
	-03 (NIPH)	-02 (ICL)	-03 (Reserve)
MU1	☐	☐	☐
NU1	☐	☐	☐
MU2	☐	☐	☐
NU2	☐	☐	☐
MU3	☐	☐	☐
NU3	☐	☐	☐
MU4	☐	☐	☐
NU4	☐	☐	☐
MU5	☐	☐	☐
NU5	☐	☐	☐
MU6	☐	☐	☐
NU6	☐	☐	☐
MU7	☐	☐	☐
Start of defrost at 4ºC (overnight)	(24 hours): _____ . _____		
Start of defrost at room temperature (30 min before aliquoting)	(24 hours): _____ . _____		
Time of aliquotting next day:	(24 hours): _____ . _____		

Day 7 night and Day 8 morning Urine sampling

Did the family bring the following urine samples to clinic (Tick all that apply):

- ☐ Night before sample
- ☐ Morning sample

How had the parents stored 'Night before sample' before coming to clinic:

☐ Stored at room temperature

☐ Stored in fridge

☐ Stored in freezer

How had the parents stored 'morning sample' before coming to clinic:

☐ Stored at room temperature

☐ Stored in fridge

☐ Stored in freezer

Action	Time performed (24 hours)	
Time of bedtime urine collection		
Time of morning urine collection		
Time of transfer into fridge at clinic		
Time of new urine collection (if needed):		
Time of aliquotting urine		
Time of freezing of urine aliquots to -80°C		

Urine Aliquots made (Tick all that apply):

☐ NUx-01	☐ MUx-01	☐ CUx-01
☐ NUx-02	☐ MUx-02	☐ CUx-02
☐ NUx-03	☐ MUx-03	☐ CUx-03
☐ NUx-04	☐ MUx-04	☐ CUx-04
☐ NUx-05	☐ MUx-05	☐ CUx-05
☐ NUx-06	☐ MUx-06	☐ CUx-06
☐ NUx-07	☐ MUx-07	☐ CUx-07

Comments on urine processing (Eg. Any deviation from protocol)

_____
_____
_____
_____
_____
_____

☐ Buccal swabs collected (participating cohorts)

Sample Data Sheet (Child Panel Week 2)

Fieldworker: _____

Day of week: _____

Date: ☐☐ ☐☐ 2 0 ☐☐

(day) (month) (year)

Helix ID: _____

Blood sampling

Vacutainers collected (Tick all that apply):

- ☐ EDTA,
- ☐ Tempus,
- ☐ Plastic serum vacutainer,

Action	Time performed (24 hours)	
Blood sampling (venopunture)		
Start of Centrifugation of EDTA tube		
Freezing of aliquots from EDTA tube to -80°C		
Freezing Tempus tube to -80°C		
Start of centrifugation of plastic serum tube		
Time of freezing of serum aliquots to -80°C		

Blood Aliquots made (Tick all that apply):

- ☐ Blood Smear
- ☐ P-01
- ☐ P-02
- ☐ P-03
- ☐ P-04
- ☐ BC-01
- ☐ RC-01
- ☐ SP-01
- ☐ SP-02
- ☐ SP-03

Comments on blood processing (Eg. Any deviation from protocol)

_____
_____
_____

Weekly Urine samples(Day 1 – Day 7 morning)

Sample	Aliquots made (tick all obtained)		
	-01 (NIPH)	-02 (ICL)	-03 (Reserve)
MU1	☐	☐	☐
NU1	☐	☐	☐
MU2	☐	☐	☐
NU2	☐	☐	☐
MU3	☐	☐	☐
NU3	☐	☐	☐
MU4	☐	☐	☐
NU4	☐	☐	☐
MU5	☐	☐	☐
NU5	☐	☐	☐
MU6	☐	☐	☐
NU6	☐	☐	☐
MU7	☐	☐	☐
Start of defrost at 4ºC (overnight)	(24 hours): _____ . _____		
Start of defrost at room temperature (30 min before aliquoting)	(24 hours): _____ . _____		
Time of aliquotting next day:	(24 hours): _____ . _____		

Day 7 night and Day 8 morning Urine sampling

Did the family bring the following urine samples to clinic (Tick all that apply):

☐ Night before sample

☐ Morning sample

How had the parents stored 'Night before sample' before coming to clinic:

☐ Stored at room temperature

☐ Stored in fridge

☐ Stored in freezer

How had the parents stored 'morning sample' before coming to clinic:

- ☐ Stored at room temperature
- ☐ Stored in fridge
- ☐ Stored in freezer

Action	Time performed (24 hours)	
Time of night before urine collection		
Time of morning urine collection		
Time of transfer into fridge at clinic		
Time of new urine collection (if needed):		
Time of aliquotting urine		
Time of freezing of urine aliquots to -80°C		

Urine Aliquots made (Tick all that apply):

☐ NUx-01	☐ MUx-01	☐ CUx-01
☐ NUx-02	☐ MUx-02	☐ CUx-02
☐ NUx-03	☐ MUx-03	☐ CUx-03
☐ NUx-04	☐ MUx-04	☐ CUx-04
☐ NUx-05	☐ MUx-05	☐ CUx-05
☐ NUx-06	☐ MUx-06	☐ CUx-06
☐ NUx-07	☐ MUx-07	☐ CUx-07

Comments on urine processing (Eg. Any deviation from protocol)

_____
_____
_____
_____
_____
_____

- ☐ Hair collected
- ☐ Buccal swabs collected? (participating cohorts)