ACTION urine and buccal DNA collection protocol

This protocol describes urine and buccal cell collection procedures in children for inclusion into our "ACTION-biomarker" studies. The parents of 6 to 12 year old children, often twins, received a letter detailing the aim of the project and asking them to participate. This letter included informed consents (IC) forms for participation in the ACTION (Aggression in Children: unravelling gene-environment interplay to Inform Treatment and InterventION strategies) project. After receiving the signed ICs or after verbal confirmation of participation via telephone (with agreement to send signed ICs by mail) the collection packages for urine and buccal cell collection were send to the participating families.

The collection packages included all the necessary materials for urine and buccal cell collection, including cover letters for each of the packages, instructions, a urine collection form (to collect information about date and time of urine collection as well as general information about current health and medication use) and also included a survey booklet with i.e. the CBCL (Child Behavior Check List) (**Figure 1**). Once urine and buccal swabs had been collected an appointment was made with the parents to collect the materials. Families received €20.- in gift certificates as a token for their participation, parents were asked to sign as proof of receiving these certificates.

Urine samples were transported in a mobile freezer unit (-18^{o}C) to the laboratory for storage (-80^{o}C) until such time the analyses could begin. Urine samples were aliquoted for biomarkers and metabolomics studies. Buccal samples were stored for DNA extraction and zygosity assessment in twins, using the GSA array. Information on zygosity was reported back to the parents of twins. DNA samples were also used for epigenetic testing on the Illumina EPIC array.

The urine collection protocol

1. The first morning urine was collected as described in the 'urine collection instruction form' (**Appendix 1**). Urine should have been collected on the first morning on which the child had not been to the toilet at night, wet the bed, or was suffering from an infection, the flue or the common cold.
2. Following the instruction manual for the uritainer (**Appendix 2**), the uritainer was placed on the toilet seat.
3. The child urinated in the uritainer.
4. The parent noted the time of urination on the urine collection form (**Appendix 3**).
5. The parent transferred the urine from the uritainer to the urine sample tubes. For each child four tubes were provided. The tubes were not allowed to exceed the limit of 12ml of urine. With a minimum of 1ml of urine for 2 of the tubes and 10ml of urine for the other 2 tubes.
6. The urine tubes were then placed in the protective blister. For each child 2 protective blisters were provided, each protective blister could hold up to two urine tubes.
7. The samples tubes in the protective blister were placed in the home freezer (-18^{o}C) a.s.a.p.
8. The parent noted the time of freezing the samples on the urine collection form, additionally, the parent filled out the remainder of the urine collection form, which consisted of information about child, the type of freezer used, any comments with regards to the urine collection, questions for girls only with regards to their potential menarche and information about the child's current health and medication use.

Figure 1. Materials included in urine (**a**) and DNA (**b**) packages

Figure 1a. Materials included in the urine collection package.

Each package included: 1x cover letter, 1x uritainer, 4x urine collection tube, 2x protective blister, 1x instruction for urine collection incl. instructions for uritainer use, 1x urine collection form and 1x survey booklet to be filled out by one of the parents about the child(ren).

Figure 1b. Materials included in the buccal cell collection package.

Each package included: 1x cover letter, 16x sterile cotton swaps, 4x tubes with buffer for storing buccal cell samples and 1x instructions for buccal cell collection.

Buccal cell collection protocol

Participants were instructed to collect buccal (epithelial) cells twice per day on 2 consecutive days: in the morning (prior to breakfast) and at evening (prior to dinner). Before buccal cell collection participants were asked not to eat, brush their teeth or gargle or rinse their mouth. At each collection point the participants needed to use 4 cotton swaps, each cotton swap needed to be used for 10-20 seconds on a specific area of the mouth while applying light pressure:

1 cotton swap to be used on the inside of the upper lip and gums of the upper jaw
1 cotton swap to be used on the inside of the lower lip and gums of the lower jaw
1 cotton swap to be used on the inside of the left cheek
1 cotton swap to be used on the inside of the right cheek

After swabbing participants placed the cotton swaps, tip-down in the provided tubes pre-filled with buffer (see Meulenbelt et al., Am J Hum Genet. 1995, 57(5):1252-4.). All four swabs needed to be placed in the same tube each collection time.

Appendix 1 – urine collection instruction form (adapted and translated from Dutch)

We would like to ask you to collection urine for our research project. This urine material can be used to study metabolites. Metabolites are metabolic products and may provide insight into the functioning of a person. As metabolite concentrations depend on the time of day, we ask you to collect your child's morning urine. Urine collection can be achieved in a straightforward manner by use of the enclosed '*uritainer*'. The exact instructions follow below.

Necessary for urine collection:

1. uritainer; a toilet holder for collecting the urine
2. 4 small tubes; for the storage of the urine
3. 2 plastic blisters; to collect the urine tubes during transport
4. Urine collection form

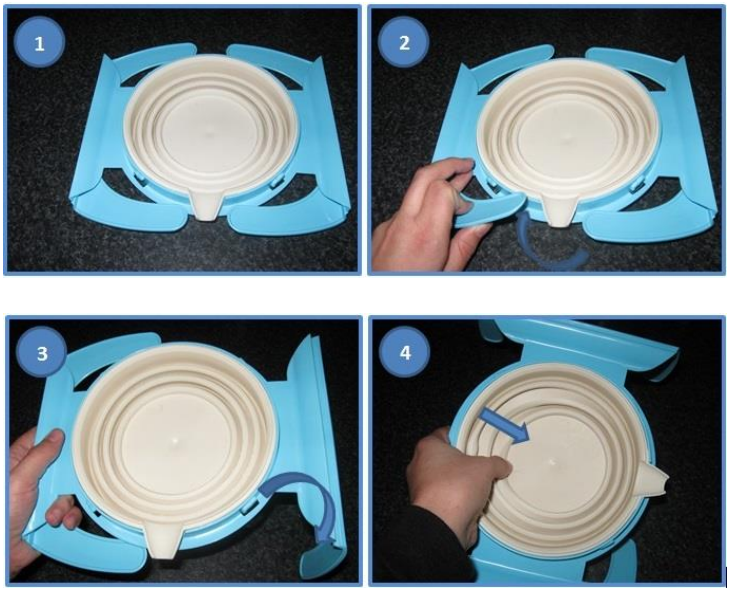
Collection:

1. Once your child has awakened in the morning you can collect the first morning urine. ***ATTENTION*:** collect the urine before your child has washed, soap residue and use of moist cloths could influence our measurements
2. Take the uritainer from the plastic wrapper and place it on the toilet (**Appendix 2**)
3. Have your child urinate into the uritainer
4. Take the uritainer from the toilet and pour the urine into the included small tubes (the uritainer has a pouring spout)
    a. We ask you to fill all the tubes. Please fill two tubes with a *minimum* of **10 ml** and the other two tubes with a *minimum* of **1 ml** (unable to fill the tubes with these minimums? Please contact us for a new collection kit!). ***ATTENTION:*** do not overfill the tubes (*maximum* of **12 ml** per tube; e.g., the top line). Urine expands during freezing and overfilled tubes might crack or open under pressure.
5. Note the time of urine collection on the urine collection form (**Appendix 3**).
6. Place the urine tubes in the plastic blisters to prevent potential leaking or damage.
7. Store the collected urine in your freezer or the freezer compartment of your fridge!
8. Note the time at which the urine was placed in the freezer on the urine collection form and fill out the remainder of this form (**Appendix 3**).

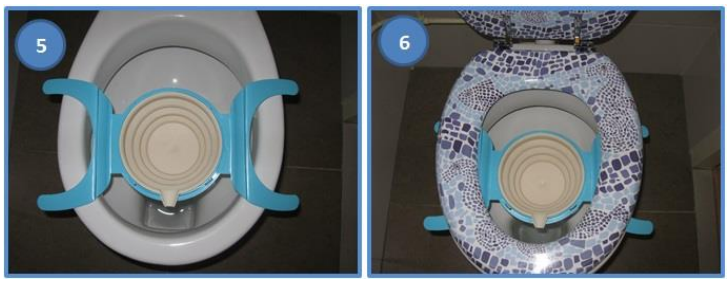
The remaining urine can be flushed and the uritainer may be recycled.

Appendix 2 – instruction manual uritainer (translated from Dutch)

1. Take the uritainer out of the plastic wrapper (***Figure 1***)
2. Lift the brackets from the edge (***Figure 2***) and fold down the brackets (***Figure 3***).
3. Push the white drip tray down until it is fully extended (***Figure 4***).



4. Place the uritainer under the toilet seat. Pay attention to the placement of the uritainer; make sure it is placed just right for the collection of urine (***Figure 5***).
5. 5. Clamp the uritainer between the toilet and the toilet seat to prevent shifting (***Figure 6***).



Appendix 3 – urine collection form (translated from Dutch)

Urine collection:

Date (dd/mm/yyyy): ……/……/……

Time urine collection (hh/mm): ……/……

Time urine in freezer (hh/mm): ……/……

Remarks with regards to urine collection or freezing:

What type of freezer do you have?

☐ freezer as compartment in the fridge

☐ freezer separate from the fridge

Other remarks with regards to urine collection or freezing?

Just for girls:

	No	Yes
Is your daughter menstruating?	☐1	☐2
Is your daughter menstruating at the time of urine collection?	☐1	☐2

Health and medication use:

	No	Yes
1. Does the child have a chronical physical condition or physical disability?	☐1	☐2
2. Does the child have a condition or disability which **severely impacts daily** functioning, despite the potential use of aids or medication?	☐1	☐2
3. Does the child currently have any infections (e.g., toothache, infected eye, urinary tract infection)?	☐1	☐2
4. Does the child currently have a (childhood) disease such as (stomach)flue or chicken-pox?	☐1	☐2

5. If you answered question 3 or 4 with '*yes*', could u specify the type of infection or illness and when this started?

Type of infection and/or (childhood) disease	Started at (date [dd/mm/yyyy])

	No	Yes
6. Does the child use any medication **at this moment**?	☐1	☐2

7. If you answered question 6 with 'yes', could you specify the type of medication, how often this is used and how long this has been used?

Medicine	Condition	Frequency	Used since (date [dd/mm/yyyy])

8. Does the child use any vitamin supplements **at this moment**? No ☐1 Yes ☐2

9. If you answered question 8 with 'yes', could you indicate the type of vitamin supplement, how often they are used and for how long they have been used?

Type supplement (e.g., brand name or description of contents)	Frequency	Used since (date [dd/mm/yyyy])

10. Could you indicate for each of the following conditions whether they are applicable to your child?

	No	Yes
a. asthma, chronic bronchitis or CARA	☐1	☐2
b. inflammation of the paranasal sinuses, forehead cavity or maxillary sinus	☐1	☐2
c. severe skin disease or eczema	☐1	☐2
d. severe bowel disorders, longer than 3 months	☐1	☐2
e. chronic cystitis	☐1	☐2
f. back disorder of persistent nature, longer than 3 months	☐1	☐2
g. epilepsy	☐1	☐2
h. serious heart defect	☐1	☐2
i. diabetes	☐1	☐2
j. malignant condition or cancer	☐1	☐2
k. liver disease or liver cirrhosis	☐1	☐2
l. severe kidney disease	☐1	☐2
m. joint inflammation or chronic rheumatism, lasting more than 3 months	☐1	☐2
n. deaf or very hard of hearing	☐1	☐2
o. blind or very visually impaired	☐1	☐2
p. spastic	☐1	☐2
q. disorder of the musculoskeletal system (orthopedic disorder)	☐1	☐2
s. allergies	☐1	☐2
type of allergies:……		
t. other **serious** congenital or **serious** long-term conditions	☐1	☐2
description:……		
11. Is the child treated for one of the disorders listed under question 10, or does the child have to check with a specialist at least once a year?	☐1	☐2

12If you answered any of the above health questions (**questions 1 to 11**) with '*yes*', could you give a brief explanation below? (e.g. a further explanation about the type of disorder or disability).

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