Protocol Full Title:

Optimization of sampling for molecular microbial research on ocular surface samples and building a framework for comparing current sequencing results using different extraction protocols.

Protocol Acronym/short title:

EPSO

Version and date of protocol:

v 7.0 dd 01-02-2021

Funding:

Name: LRD/ KU Leuven: EFW-FOPRD1-O2010 (Perdaens Eye Research Fonds)
Address: Waaistraat 6, 3000 Leuven

Name: UZ Leuven: Fonds Academisch onderzoek, S62672
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Signatures

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Principal Investigator Date: 01.02.2021

Name: Dr. H. Delbeke

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Study Synopsis

Title of clinical trial	Optimization of sampling for molecular microbial research on ocular surface samples and building a framework for comparing current sequencing results using different extraction protocols.
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Funding	LRD/ KU Leuven: EFW-FOPRD1-O2010 (Perdaens Eye Research Fonds) UZ Leuven: Fonds Academisch onderzoek S62672
Principal Investigator	Dr. H. Delbeke
Sponsor	University Hospitals Leuven Herestraat 49 3000 Leuven
Purpose of clinical trial	Optimization of ocular sampling for microbial research and to provide a framework for the interpretation of research results for ocular surface microbiome studies based on differences in DNA extraction procedures of complex microbial communities.
Trial Design	Exploratory study using biological samples
Summary of eligibility criteria	• ≥ 18 years old • Willing to undergo sampling of the conjunctiva • Fluent in written and verbal Dutch • Capable of giving informed consent

Study protocol EPSO v 7.0 dd 01.02.2021:
Optimization of sampling for molecular microbial research on ocular surface samples and building a framework for comparing current sequencing results using different extraction protocols.

Version and date of final protocol	v 7.0 dd 01.02.2021

1. Background, rationale and novelty

The human microbiome is the collective genomic information of the community of commensal, symbiotic and pathogenic microorganisms that share our body space. They play a role in metabolism, homeostasis and maturation of the immune system (1)

Since the publication of the Human Microbiome Project in 2008, a lot of research on the presence of a core microbiome on different body parts such as gut, skin and mouth has been executed. More and more evidence is emanating that a dysbiotic microbiome can induce a variety of autoimmune and inflammatory diseases. Especially the relationship between the intestinal microbiome and different inflammatory diseases has been widely investigated (2).

Since culture-based methods only reflect a subset of the microorganisms present in complex human associated microbial communities, it is necessary to additionally use culture independent methods to further investigate the presence of a "core" microbiome per microbial habitat (3). High-throughput sequencing, such as amplicon-based studies or metagenomic studies are the current way to go. Targeted amplicon studies, such as 16S rRNA amplicon sequencing, focus on marker genes and use these markers to reveal the composition and diversity of the microbiota. Part of the 16S rRNA gene is amplified by PCR with universal primers that recognize highly conserved regions of the gene. This region is then further sequenced. Metagenomic studies, as shotgun whole genome sequencing (WGS) use, instead of universal primers, random primers to sequence overlapping regions of the complete genome (4, 5)

Several studies have described the presence of a core ocular surface microbiome, but the difference in methods used makes them quite difficult to compare.
In terms of used swabs, some studies use sterile cotton swabs (6-11) others nylon flocked swabs (2). Also, the use of **anesthetics** is subject for discussion. Some researchers use anesthetics as this would improve the sampling on subjects (11, 12). However, since Shin et al. (9) published a significant higher alpha diversity when no anesthetic was used, most sampling was performed without anesthesia. The latter publication did however not provide any information on timing

between application and sampling. Indeed, the described effect could also be due to the diluting effect of the drop itself. In any case, when using a topical anesthetic drop, the firmness of the sampling can be considered influenced. Possibly there is a vertical stratification with the presence of transient species in the more superficial layers and a more resident commensal microbiota in the deeper layers (7).

To compare the effect of anesthetic drops, we decided to sample volunteers undergoing general anesthesia for any type of surgery. The right and left eye of one person will be randomized for a drop of artificial tears (Thealoz duo®, Théa Pharma, Clerrmont – Ferrand, Cedex 2, France) or a drop of topical anesthesia (Minims® Oxybuprocaine Hydrochloride 0.4%, Bausch and Lomb, Aubenas, Frankrijk). One drop of Thealoz duo® has the same weight as one drop of Minims® Oxybuprocaine Hydrochloride 0.4%, (both 0,02 g), this makes the diluting effect comparable. The executer will be blinded. The rationale for general anesthesia is that the sampling pressure/depth will be similar with or without topical anesthesia since the executer won't be influenced by the patients reaction. By comparing both eyes of one person, the immunological and genetic background will be the same. Moreover, Shalabi et al. showed a high level of concordance in microbiome composition in the same-person eyes (13). The effect of environmental factors, such as the effect of repeatedly sleeping on one side, will be minimalized by randomization. To have the possibility to take the most obvious confounding factors in account, subjects will be asked to fill in a small questionnaire *(see addendum A).*

In gut and oral microbiome research, it is currently acknowledged that the used **DNA extraction method** plays a crucial role in the recovery of microbial taxa (14-16). However, researchers focusing on less studied human microbial niches, like the ocular surface, seem not yet to be aware of the impact thereof. The ideal extraction protocol should reflect the microbial richness and diversity present on the eye as adequate as possible since the DNA extraction method affects the observed species diversity downstream. We aim at comprehensible integration of the results from the scarce microbiota studies published in this field, by

comparing different DNA extraction procedures used in these studies. By sampling both eyes of 7 volunteers on 7 different points in time, 6 extraction protocols will be compared, of which one with and without human depletion. *(fig.1).* In an attempt to reduce possible confounding factors of repetitive sampling, sampling will not be performed on consecutive days and all samples from the 7 subjects from a certain day will be tested with a different extraction protocol in each subject (as outlined in the below overview).

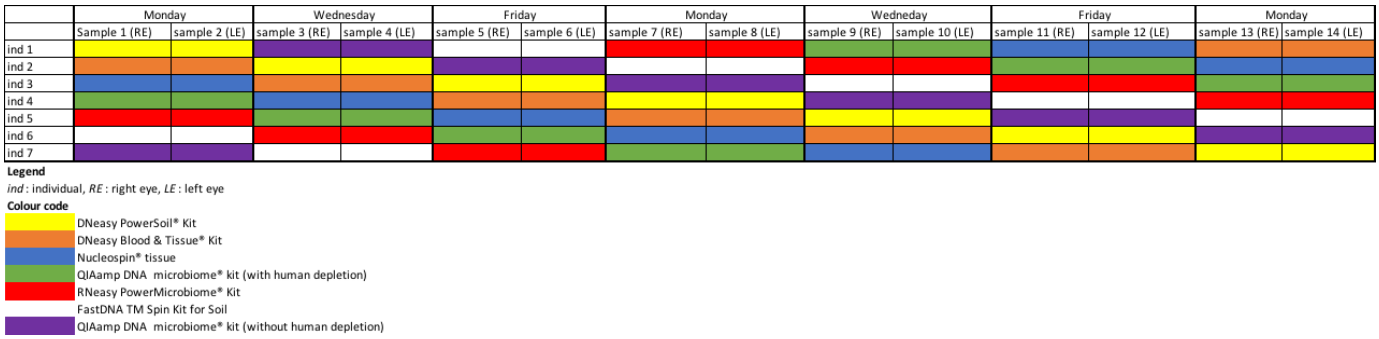


Figure 1

Although the immunological and genetic background in one person are the same and Shalabi et al. showed a high level of concordance in microbiome composition in the same-person eyes (13) , the environmental factors may differ between eyes. First of all, patients are known unilateral eye rubbers, with sometimes clinical consequences such as keratoconus (17). Secondly, repeatedly sleeping on one side can have an effect on the ocular surface microbiome as well due to rubbing of the eyelid against the pillow and nocturnal eyelid eversion (18). To further clarify the effect of environmental factors on both eyes within one individual, the 7 volunteers mentioned before will be asked to fill in a questionnaire *(cfr. addendum A).*

The **ambition** of this study is to make our research done in this area more robust since it will determine our method used in each future project.

2. Trial objectives and design

We aim at clarifying 1) if the reported effects of the use of anaesthetic drops (9) are due to dilution of samples or not, 2) how different DNA extraction protocols distort our view on ocular

surface microbiota composition and 3) the degree of similarity between left and right eye in the same person.

2.1 Preparation, organization and management of this exploratory study

Part 1: Effect of the use of anaesthetic drops on ocular microbial composition using 16S rRNA sequencing.

Study cohort:

Thirty volunteers undergoing general anaesthesia for any type of surgery will be informed about the study upon their consultation at the Ophthalmology Department of the University Hospitals Leuven, Belgium. In case a candidate is interested in participating, he/she will be informed about the study goals and protocols by *dr. H. Delbeke* or the responsible clinical research coordinator *Inge Vriens* during a personal conversation. Each volunteer will receive an information letter to take home.

Study procedure:

After signing the informed consent, each patient will be asked to fill in a questionnaire *(cfr. addendum A).*

All participants will undergo conjunctival sampling of both eyes **under general anaesthesia.** The research coordinator or operating room nurse will applicate one drop of preservative free artificial tears to one eye Thealoz duo®, Théa Pharma, Clerrmont – Ferrand, Cedex 2, France) and one drop of preservative free anaesthetics (Minims® Oxybuprocaine Hydrochloride 0.4%, Bausch and Lomb, Aubenas, Frankrijk) to the other eye. A timed 5 minutes will be in between application of the drop and the sampling. The sampling will be blinded for the executer and will be performed by myself, Heleen Delbeke, and/or residents and/ or medical students after proper training of correct sampling. Each eye will be sampled with a single, sterile, nylon, flocked swab (FLOQSwabs®; Copan, Brescia, Italy). The

sampling will be done from the nasal to temporal inferior conjunctival sac, the swab will be swirled in the opposite direction of the sampling itself. The swabs will be placed in a microcentrifuge tube and immediately frozen at −20 °C for a maximum period of 2 weeks, after which they will be transferred to a −80 °C freezer until further processing. DNeasy PowerSoil® Kit will be used for DNA extraction. This extraction protocol is chosen based on its frequency used in ocular surface microbiome research. The extracted DNA will be analysed using the V4 region of the 16S rRNA gene. All steps in between sample taking and sequencing will be executed at the REGA institute for medical research or department of Biochemistry and Microbiology of the University of Gent. The sequencing itself will be done at a sequencing facility.

Part 2: Clarification on how different DNA extraction protocols distort our view on ocular surface microbiota composition.

Study cohort:

Seven volunteers will be recruited using a poster at the Ophthalmology Department of the University Hospitals Leuven, Belgium and at the faculty of medicine, Catholic University Leuven, Belgium (*cfr. addendum B*). In case a candidate is interested in participating, he/she will be informed about the study goals and protocols by *dr. H. Delbeke* or the responsible clinical research coordinator *Inge Vriens* during a personal conversation. Each volunteer will receive an information letter to take home.

Study procedure:

After signing the informed consent, all eligible participants will be asked to fill in a small questionnaire *(cfr. addendum A),* after which they will undergo conjunctival sampling on 7 different points in time *(figure 1)*. Based on the results of the first part of the study we will not use anaesthetic drops.

There is no need for general anaesthesia. The sampling will be performed by myself, Heleen Delbeke, and/or residents and/ or medical students after proper training of correct sampling. Each eye will be sampled with a single, sterile, nylon, flocked swab (FLOQSwabs®; Copan, Brescia, Italy). The sampling will be done from the nasal to temporal inferior conjunctival sac,

the swab will be swirled in the opposite direction of the sampling itself. The swabs will be placed in a microcentrifuge tube and immediately frozen at −20 °C for a maximum of 2 weeks, after which they will be transferred to a −80 °C freezer until processing. Six extraction protocols will be compared, of which one with and without human depletion. The extracted DNA will be analysed using the V4 region of the 16S rRNA gene. All steps in between sample taking and sequencing will be executed at the REGA institute for medical research or department of Biochemistry and Microbiology of the University of Gent The sequencing itself will be done at a sequencing facility.

Part 3: What is the degree of similarity between left and right eye in the same person.

Study cohort and procedure:

Part 3 is based on the questionnaire and sampling results of the ***seven subjects*** used in ***part 2***.

3. Selection and withdrawal of subjects

3.1 Inclusion criteria

Part 1: Effect of the use of anaesthetic drops on ocular microbial composition using 16S rRNA sequencing.

Subject has to be older than 18 years old

Subject undergoes general anaesthesia for whatever reason

The subject is willing to undergo sampling of the conjunctiva of both eyes

The subject is fluent in written and verbal Dutch

The subject is capable of giving informed consent

Part 2: Clarification on how different DNA extraction protocols distort our view on ocular surface microbiota composition.

Subject has to be older than 18 years old

The subject is willing to undergo sampling of the conjunctiva of both eyes on 7 different time points

The subject is fluent in written and verbal Dutch

The subject is capable of giving informed consent

3.2 Exclusion criteria

- Allergy to Oxybuprocainehydrochloride
- Ocular surface disease
- Medication usage in one eye only

3.3 Expected duration of trial

Inclusion of subjects is planned between August the 15th 2019 and the end of December 2020.

4. Trial procedures

4.1 Summary of visits

Part 1: Effect of the use of anaesthetic drops on ocular microbial composition using 16S rRNA sequencing.

The conjunctival samples will take place at time of the planned general anesthesia. No additional visits are necessary.

Part 2: Clarification on how different DNA extraction protocols distort our view on ocular surface microbiota composition.

The conjunctival samples will take place at the Ophthalmology Department of the University Hospitals Leuven, Belgium on 7 different points in time. Sampling will take less than 5 minutes. To compensate for travel expenses and time invested, each participant will receive at

the end of their participation a voucher of 50 euros.

4.2 Supervision and responsibilities

The study will be performed under the supervision of dr. H. Delbeke. The subjects will be recruited at the Ophthalmology Department of the University Hospitals Leuven, Belgium and at the faculty of medicine, Catholic University Leuven, Belgium. The sampling will be performed by myself, Heleen Delbeke, and residents and/ or medical students. Each study subject will sign an informed consent. The participating investigators will perform their part of the study fully in accordance with the terms of the Protocol, the applicable national and international laws (amongst others: the European General Data Protection Regulation of May 25, 2018) and apply and adhere to regulations and rules as, amongst others, the Declaration of Helsinki (2008) and ICH GCP Guidelines.

4.2.1 Insurance

In accordance with the Belgian Law relating to experiments on human persons dated May 7, 2004, UZ/KULeuven shall assume, even without fault, the responsibility of any damages incurred by a Study Patient from the UZ/KU Leuven site and linked directly or indirectly to the participation to the Study, and shall provide compensation therefore through their insurance (Amlin Corporate Insurance, polisnr. 299.053.700, Vanbreda Risk & Benefits, Plantin en Moretuslei 297, 2140 Antwerp).

4.2.2 Informed consent

The Participating Site acknowledges that the Study can and will be conducted only on the basis of prior informed consent by the Subjects, or their legal representatives, to participate in the Study. The Participating Site shall obtain a signed informed consent form (ICF) for all patients prior to their enrollment and participation in the Study in compliance with all applicable laws,

regulations and the approval of the local Ethics Committee, if required. The Participating Site shall retain such ICFs in accordance with the requirements of all applicable regulatory agencies and laws.

5. Assessment of efficacy

Not applicable

6. Assessment of safety

When sampling is not performed under general anesthesia, a slight discomfort can be perceived.

When topical anaesthetics are being used, a transient stinging and blurring of the vision after instillation can occur. When used in larger amounts, epitheliopathy of the cornea can be noticed. This is only rare in the case of one drop. Systemic absorption will be reduced by asking the patient to gently close the lids and to press on the medial canthus for a minute following the instillation of the drops. There are no known interactions with other medical products. It will not be used during pregnancy or lactation, nor will it be used in patients with a known hypersensitivity to the product.

After sampling the conjunctiva, a minor bleeding can occur in the conjunctival sac which can induce a feeling of bruising of the tissue. Scarring due to the intervention is extremely rare.

7. Statistics

7.1 Sample size

Since this is an **exploratory study**, we are not able to perform proper sample sizing.
For the first part, we aim at sampling 30 patients (60 samples) taking into account a maximal loss of 10 samples due to processing issues. Taking this loss into account, we will be able to

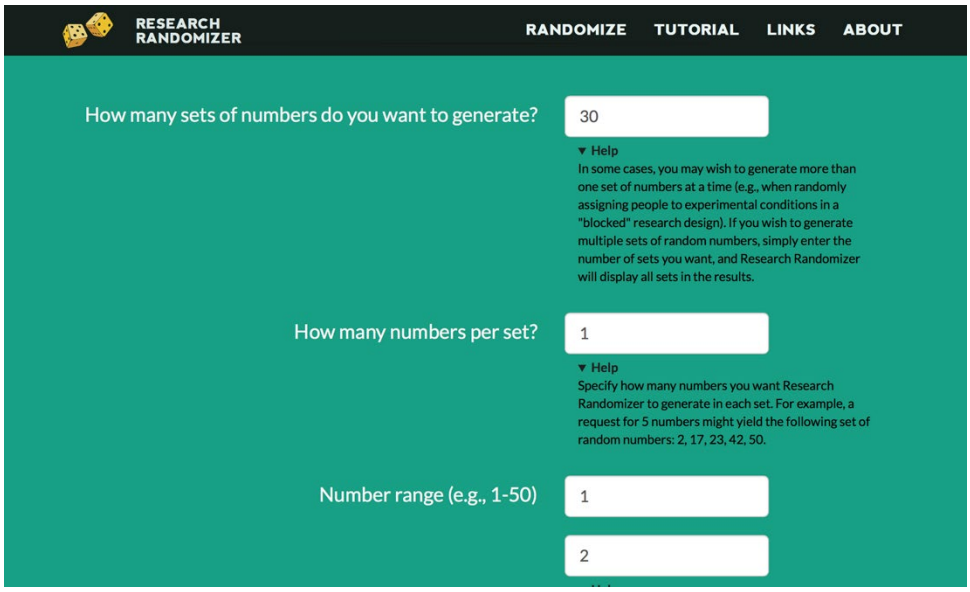
process 50 samples to elucidate if the use of anesthetic drops has an effect on the sequencing results.

For the second part, we wish to sample 7 volunteers so we can compare 7 extraction protocols on different subjects on non-consecutive days. Each protocol will be tested on a sample form a different individual on a different day *(figure 1)* to minimize possible confounding factors. In gut and oral microbiome research, samples are homogenized to make comparing of different extraction protocols possible. In ocular surface samples, the retrieved sample is to small for homogenization.

7.2 Randomization

A 30-patient **randomization list** will be generated by our clinical research coordinator using https://www.randomizer.org, where "1" will be a drop of preservative free anaesthetics in the right eye and "2" a drop of preservative free anaesthetics in the left eye.

By using this software, we are able to automate the randomization, this to reduce bias due to manual randomization. When analysing our results, we will search for other biases due to randomisation, which we will be taken into account.



7.3 Analysis

The sequences will be analysed using the latest bioinformatics tools (like Dada2) and analysis will be done with the R software. The alpha diversity will be evaluated using Shannon's Diversity index and Simpson's diversity index; the beta diversity will be assessed by using Bray-Curtis dissimilarity, Jaccard distance and permutational multivariate analysis of variance. Differences will be considered statistically significant with a P-value ≤ 0.05. (19, 20)

8. Quality assurance

To assure maximal quality and reproducibility, the trial protocol will be followed rigorously.

9. Direct access to source data and documents

Only data gathered in the context of the trial will be used.

10. Ethics and regulatory approvals

The trial will be conducted in compliance with the principles of the Declaration of Helsinki (2008), the principles of Good Clinical Practice and in accordance with all applicable regulatory requirements. This protocol and related documents will be submitted for review to Ethics Committee of the University Hospitals Leuven.

The Study will be conducted only on the basis of prior informed consent by the Subjects to participate in the Study. We shall obtain a signed informed consent form (ICF) for all patients prior to their enrollment and participation in the Study in compliance with all applicable laws, regulations and the approval of the (local) Ethics Committee, if required. We shall retain such ICFs in accordance with the requirements of all applicable regulatory agencies and laws.

11. Data Handling

We shall treat all information and data as confidential and shall not disclose such information to any third parties or use such information for any purpose other than the performance of the Study. The collection, processing and disclosure of personal data, will comply with applicable personal data protection and the processing of personal data (the European General Data Protection Regulation of May 25, 2018). We will protect the data from disclosure outside the research according to the terms of the research protocol and the informed consent document. The subject's name or other identifiers will be stored separately from their research data and replaced with a unique code to create a new identity for the subject.

12. Data Management

All data will be analyzed and stored in a coded fashion, with a unique anonymous identifier for every subject. In the future, these data could be used in the context of this project, and for related projects.

13. Publication Policy

Any publication will be submitted to all co-authors at least thirty (30) days prior to submission or disclosure.

14. Financial Aspects

No commercial conflict of interest is related to this study.
For the first part of this study, conjunctival sample will take place at time of the planned general anesthesia. No additional visits are necessary. Volunteers do not receive a financial compensation for their participation.
For the second part, the subject needs to come on 7 different points in time for sampling. Sampling will take less than 5 minutes. The visit will not be charged. If there were to be additional medical expenses (medication and visits) arising from participation in the study,

they will not be charged to the volunteer. To compensate for travel expenses and time invested, each participant will receive at the end of their participation a voucher of 50€.

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16. Addenda

Addendum A: questionnaire to be filled in by all subjects