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TITLE: Feasibility of CRISPR-Cas9-Based In Vitro Drug Target Identification for Personalized Prostate Cancer Medicine

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14. ABSTRACT This study tests the feasibility of using CRISPR-Cas9 to introduce patient-derived clonal truncal mutations into prostate cell lines in order to study their potential druggability. We aimed to establish a positive control cell line by introducing a known <i>PIK3CA</i> mutation that is druggable using available PI3K pathway inhibitors. However, using immortalized normal prostate epithelial cell line RWPE-1 we had significant problems getting CRISPR working efficiently. We suspected this being due to the chromatin status of <i>PIK3CA</i> locus in these cells, as the gene in very lowly expressed. Thus, we have moved on to creating the actual patient-derived clonal truncal mutations in this cell line. Currently we are in the process of creating clonal cell lines and hope to be able to sequence-verify targeted mutagenesis and then proceed to drug sensitivity testing.					
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1. INTRODUCTION

Currently there are no curative treatment options available for metastatic prostate cancer, and the treatments available can at best slow down the cancer and relieve the symptoms. Here, we take advantage of the identification of clonal truncal mutations, i.e. mutations present in all the metastatic cancer foci of a single patient with lethal metastatic disease. This study tests the feasibility of using CRISPR-Cas9 induced targeted mutagenesis together with an *in vitro* drug screening to identify potential druggable mutations among the truncal clonal mutations of a patient with metastatic prostate cancer. CRISPR-Cas9 is used to introduce patient-derived truncal passenger mutation into normal immortalized prostate cell line or a prostate cancer cell line. Clonal cell line with sequence-verified truncal mutation will be screened *in vitro* against a compound library of ~530 FDA/EDA-approved or emerging cancer-drugs. Parental non-targeted cell line will serve as a control for the drug screening. The possible off-target effects of CRISPR will be addressed through appropriate sequencing approach (e.g. Digenome-seq or Guide-seq). Ultimately, we aim to answer if the described approach should be further developed and tested for its potential use in helping clinical decision-making.

2. KEYWORDS

Metastatic prostate cancer, clonal truncal mutations, CRISPR-Cas9, knock-in, druggability, feasibility study

3. ACCOMPLISHMENTS

All major tasks and subtasks listed in the original SOW relevant to the reporting period are listed below and addressed separately.

Training-specific tasks (80% of planned completed)

Major Task 1: Training and educational development in prostate cancer research	Months		Completion
Subtask 1: Attend a scientific research workshop related to CRISPR-Cas9	1-6	Dr. Rauhala	0 %
Subtask 2: Present research at the weekly group meetings	1-24	Dr. Rauhala	100 %
Subtask 3: Attend a national scientific meeting in relevant scientific field	1-24	Dr. Rauhala	0 %
Subtask 4: Attend an international scientific meeting in relevant scientific field (e.g. AACR Annual meeting 2017)	1-24	Dr. Rauhala	100 %

Of the training-specific tasks, Dr. Rauhala has attended weekly prostate cancer research group meetings that have offered immediate support in burning technical issues when discussed with other scientists in the group. Additionally, Dr. Rauhala has organized people working with varying CRISPR projects at University of Tampere to meet and discuss their projects and share methods, experiences etc. A

discussion forum was also set-up using *slack* messaging system to provide easy access connecting between people doing CRISPR work at the campus. Dr. Rauhala has also co-organized Cancer Journal Club for PhD students and post-docs at the Faculty of Medicine and Biosciences.

In April 2017 Dr. Rauhala attended the AACR Annual Meeting in Washington, DC. This meeting helped to connect with other researchers working on CRISPR-Cas9 knock-in projects. While in Washington, DC, Dr. Rauhala visited Dr. Suk-See Lee at NIH/NIAID to have some first-hand experiences from a scientist who has successfully implemented CRISPR-Cas9 technology in targeted mutagenesis. At the annual Research Day organized at by the Faculty of Medicine and Biosciences at University of Tampere, Dr. Rauhala had a poster presentation of her project.

Mentoring-specific tasks (100% of planned completed)

	Months		Completion
Subtask 1: Implement and refine project management system consisting of: Scheduled meeting at least once per week; Written Google Document containing project goals and milestones and dated written progress notes from each weekly meeting.	1-24	Dr. Bova Dr. Visakorpi Dr. Rauhala	100 %
Subtask 2: Quarterly review of training subtask completion and overall quality of work progress using separate Google document with dated written progress notes from each quarterly discussion.	1-24	Dr. Bova Dr. Visakorpi Dr. Rauhala	100 %

Dr. Rauhala has had monthly meetings with her mentor Dr. Bova to discuss the course of the project, with brainstorming and troubleshooting regarding the various methodological issues as well as steering the project scientifically. Google Document has been implemented as primary project progress log. This log has been revised at each project meeting with new commentary made after each meeting and novel updates in between the meetings.

Research-specific tasks

The research-specific major tasks in the SOW for the first year of the project were the creation of control cell lines using CRISPR-Cas9 and performing the drug sensitivity testing and subsequent analysis for these cell lines.

Major Task 1: Creating control cell lines using CRISPR-Cas9n	Months		Completion
Subtask 1: Setting up systems for sample handling, naming, and labeling to minimize risk of cell line cross-contamination.	1-2	Dr. Rauhala Dr. Bova	50 %
Subtask 2: Setting up the CRISPR-Cas9n assay components	1-2	Dr. Rauhala	100 %
Subtask 3: Creating the CRISPR-Cas9n modified control cell lines Cell lines used: 22Rv1, RWPE-1 (ATCC)	2-6	Dr. Rauhala	0 %
Subtask 4: Validating the of target effects of CRISPR-Cas9 in control cell lines using GUIDE-sequencing	5-8	Dr. Rauhala Dr. Nykter Dr. Bova	0 %
<i>Milestone Achieved: Efficiency and specificity of CRISPR-Cas9n modifications established</i>	8	Dr. Rauhala Dr. Nykter Dr. Bova	20 %

Major Task 2: Testing the drug responsiveness of our CRISPR-Cas9n modified control cell lines	Months		Completion
Subtask 1: Running drug sensitivity testing on control cell lines	9-10	Dr. Rauhala Dr. Östling	0 %
Subtask 2: In depth analysis of the drug sensitivity data	9-12	Dr. Rauhala Dr. Bova Dr. Östling Dr. Visakorpi Dr. Nykter	0 %
<i>Milestone Achieved: Drug sensitivity testing platform validated to identify changes in drug responses caused by single mutations</i>	12	Dr. Rauhala Dr. Bova Dr. Visakorpi Dr. Nykter Dr. Östling	0 %

Major Task 3: Creating prostate cell lines with A21's truncal missense mutations using CRISPR-Cas9n	Months		Completion
Subtask 1: Creating CRISPR-Cas9n – target mutation modified cell lines Mutations created: <i>PIK3CG</i> , <i>ABCC4</i>	13-17	Dr. Rauhala	15 %

Major Task1/Subtask 1: Setting up systems for sample handling, naming, and labeling to minimize risk of cell line cross-contamination. (50% of planned completed)

During the first rounds of CRISPR work on cell lines, special thought has been given to ways of naming and organizing samples in order to keep proper track of samples and avoid cross-contamination. The sample information will be integrated to the ILSR (Integrated Life Science Research) database in the future as the first pipeline is finished and can be used to model the different inputs and variables needed in the database for the project.

Major Task 1/Subtask 2: Setting up the CRISPR-Cas9n assay components. (100% of planned completed)

Figure 1 represents the schematic flow of creating knock-in mutations using CRISPR-Cas9. The image is from Ran *et al.* (Nat Protoc. 2013;8:2281-2308). Individual steps are explained in more detail below.

sgRNA design was done using CRISPR Design Tool (crispr.mit.edu) together with Casellas lab CRISPR Tool. Of the sgRNAs designed for any targeted loci, we chose max 4 sgRNAs that had high CRISPR score, as few predicted off-targets as possible and had their cut-site as close as possible to the intended mutation site. We then analyzed the sgRNA PAM sequences for the possibility to silently mutate them in the HDR repair template in order to prevent further cutting of once-repaired locus. The chosen guides were ordered from Integrated DNA Technologies (IDT) as ssDNA oligos.

Cas9-expressing plasmid (pSpCas9(BB)-2A-Puro (PX459) V2.0) was obtained from Addgene. Instead of using Cas9n-expressing plasmid (as originally planned), we decided to use the wtCas9 as the sgRNA design turned out to be challenging for Cas9n, when we wanted to get our guides as close as possible to our target mutation site, and be able to silently mutate the PAM sequences in the HDR repair template DNAs. sgRNA were cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 as described in Ran *et al.* (Nat Protoc. 2013;8:2281-2308). ssODN HDR-repair templates were designed using the guidelines in Richardson *et al.* (Nat Biotechnol. 2016;34:339-44), i.e. asymmetric 130bp long single-

stranded donor DNAs that enhance the HDR efficiency of CRISPR-induced DSB repair. In addition to introducing the wanted single nucleotide mutation, the donor DNAs were silently mutated at the sgRNA PAM site. Desktop Genetics design tool (deskgen.com) was used to help in donor DNA design. The ssODNs were also purchased from IDT. SURVEYOR assay from IDT was used to assess the CRISPR efficiency (i.e. cutting efficiency) of a given sgRNA.

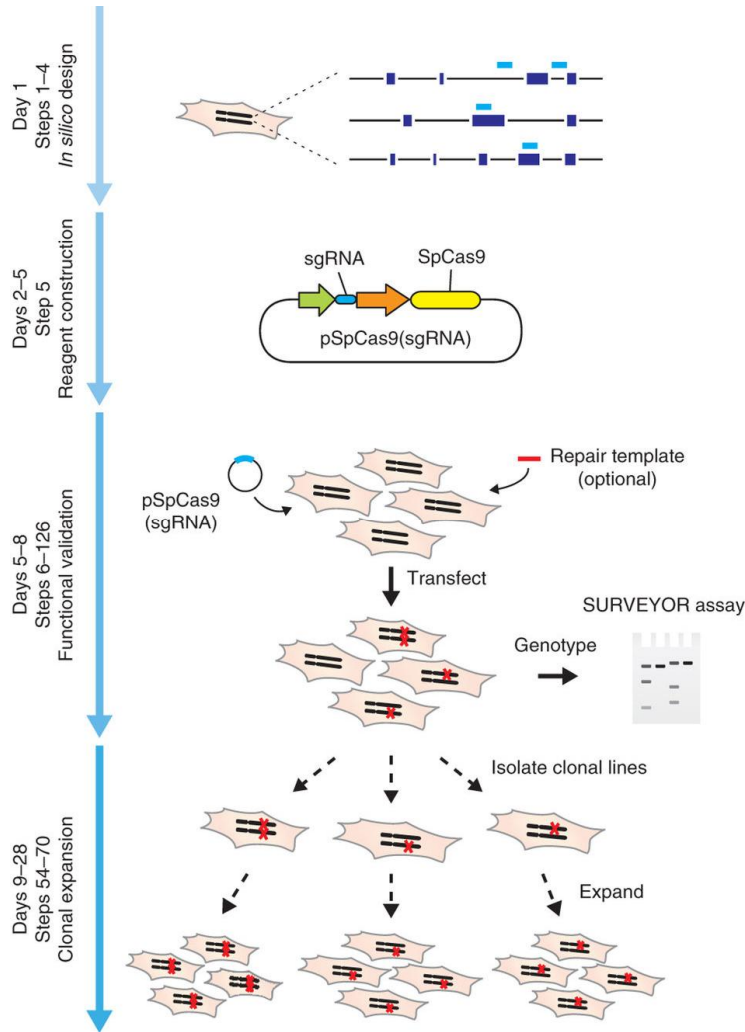


Figure 1. Approximated timeline and overview of creating knock-in mutations using CRISPR-Cas9. (Graph from Ran et al. Nat Protoc. 2013;8:2281-2308).

RWPE-1 cell line transfection efficiencies were tested using three different transfection reagents (Lipofectamine 3000, Invitrogen; Fugene, Promega; jetPRIME, PolyPLUS) together with pGFPmax plasmid that allows easy visual inspection of transfection efficiency. Based on these test we chose to transfect RWPE-1 cells with Fugene that gave us consistent >80% transfection efficiency. The used pGFPmax plasmid is only ~3,5kb in size, whereas the Cas9 and sgRNA encoding plasmid pSpCas9(BB)-2A-Puro (PX459) V2.0 is 9,2kb in size. The bigger size of the CRISPR plasmid most likely reduces the transfection efficiency to some extent, but the possible untransfected cells are then selected off using puromycin.

We have also tested the ability of our selected cell lines to form clones, i.e. start growing from single cells. RWPE-1 cells are capable of growing from single cell dilution, whereas T47D cells (carrying a *PIK3CA* mutation we intended to correct) failed to form clones when seeded at single cell /well density. We tested seeding cells at single cell density using FACS (FACS Aria Fusion, BD BioSciences) and doing manual dilution. In our tests, we were able to recover more clones from manual seeding (up to 30% of wells in 96 well plate) than from using FACS (up to 19%), implying that RWPE1 cells do not recover very well from FACS treatment. We will still try to enhance clone formation by increasing the cell density in manual dilution. This might lead to some non-clonal growth but will hopefully also increase the number of clones established. For T47D cells we will try to establish clones using conditioned media, i.e. collect media from cultured T47D cells and use that as a supplement for clonal cell growth media.

Major Task 1/Subtask 3: Creating the CRISPR-Cas9 modified control cell lines (Cell lines used: 22Rv1, RWPE-1 (ATCC). (0% of planned completed)

As a control cell line for CRISPR-induced mutagenesis combined with drug-screening, we set out to introduce known hot spot *PIK3CA* mutations into *PIK3CA* wild type immortalized normal prostate epithelial cell line, RWPE-1. This approach was chosen based on the existing PI3K pathway targeting drugs in the compound library to be used for the drug screen. RWPE-1 cell line was chosen because no mutations have been reported affecting the PI3K pathway in this cell line, whereas most prostate cancer cell lines have some genomic alterations along the PI3K pathway, thus possibly complicating identifying effects of additional mutations. The COSMIC database was searched for the most frequent *PIK3CA* affecting missense mutations. As *PIK3CA* has a pseudogene (Müller *et al.* Leuk Res. 2007;31:27-32), we omitted mutations that had a possible counterpart in the pseudogene, including the exon 10 residing mutational hotspot. We chose 4 known mutations from *PIK3CA* and designed sgRNAs against them as described above. One of the four mutations was dropped at this point, as none of the sgRNA PAM sites could be silently mutated. In the end, 2 sgRNAs for 3 different mutated loci of *PIK3CA* were selected (namely c.263G>A_p.R88Q, c.1035T>A_p.N345K and c.3140A>G_p.H1047R). In addition to introducing mutations, c.3140A>G_p.H1047R mutation present in T47D breast cancer cell line was chosen to be corrected back to wild-type using CRISPR.

sgRNA CRISPR efficiency, i.e. the ability of a given sgRNA to induce DSBs was studied first in easy-to-transfect 293T cells. sgRNA and Cas9 expressing plasmid was transfected into 293T cells and puromycin selection was started 24h after transfection to enrich for transfected cells. 72h post-transfection (48h selection) the cells were collected and DNA was amplified at the targeted loci. CRISPR cutting efficiency was determined using SURVEYOR mismatch assay (IDT) together with CRISPR Discovery Gel Kit (Advanced Analytical). CRISPR efficiencies observed in 293T cells varied between 18-55%. We did additional sequencing analysis at the CRISPR'd loci to verify the observed CRISPR action. Indeed, we observed indels at expected ratios (as compared to SURVEYOR assay) with varying indel sizes. Of the tested 2 guides per mutation the better performing (higher CRISPR efficiency) was selected to be used in RWPE-1 cells together with the HDR repair templates. RWPE-1 cells were transfected and selected as described above for 293T cells. After 48 hour selection the surviving cells were plated at 1cell/well density into 96-well plates and allowed to grow for 7-10 days after which the clone formation was determined under light microscope. Aliquot of the cell pool at the point of single cell seeding was used for SURVEYOR assay to study the CRISPR efficiency. Unfortunately, in RWPE-

1 cells we failed to observe higher than 30% CRISPR efficiencies, even though guides had shown approximately 2x higher efficiencies in 293T cells. This might reflect the chromatin status at the targeted loci, as it has been shown that transcriptionally inactive loci are more difficult to target with CRISPR than loci that are actively transcribed (Chen *et al.* NAR 2016;44:6482-92). *PIK3CA* expression level is relatively low in RWPE-1 cells, as in most prostate cancer cell lines as well, so it might be that the particular genomic locus is fairly problematic to target in these cells.

Table 1. Observed CRISPR efficiencies from SURVEYOR assay in 293T and RWPE-1 cells using different *PIK3CA* targeting sgRNAs.

sgRNA	CRISPR efficiency in 293T cells	CRISPR efficiency in RWPE-1 cells
PIK3CA c.263 #4	50	30
PIK3CA c.263 #5	42	22
PIK3CA c.1035 #1	30	10
PIK3CA c.1035 #4	55	10
PIK3CA c.3140 #9	18	4

Due to the observed low CRISPR efficiency at the *PIK3CA* loci in RWPE1 cells, combined with the expected very low rate of HDR (might be as low as 0.1%) and suboptimal (30%) clonal establishing efficiency, we decided not to pursue further creating these *PIK3CA* mutations carrying RWPE-1 control cell lines, but to move to creating our actual test cell lines carrying the truncal clonal mutations identified from a patient. Some of these mutations are present in genes that are highly expressed in our cell lines and thus we believe that they are more easily targeted by CRISPR-Cas9 than *PIK3CA*.

Major Task 1/Subtask 4: Validating the of target effects of CRISPR-Cas9 in control cell lines using GUIDE-sequencing. (0% of planned completed)

Since no clonal CRISPR'd control cell lines have been established yet, no sequencing analysis has been done.

Milestone Achieved: Efficiency and specificity of CRISPR-Cas9n modifications established. At this point of the project we can already say that CRISPR-Cas9 genome-editing efficiency in prostate cancer and normal cells is genomic locus- and cell type-specific and should be addressed separately for each locus to-be-edited. The specificity of CRISPR-Cas9 in prostate cancer and normal cells has not been addressed yet.

Major Task 2: Testing the drug responsiveness of our CRISPR-Cas9n modified control cell lines. (0% of planned completed)

Since no clonal CRISPR'd cell lines have been established yet, the drug responsiveness testing and analysis has not been done at this point. Once such cell lines are available, we will immediately move on to drug testing part of the study in collaboration with Dr. Östling at SciLife, Karolinska Institute.

Milestone Achieved: Drug sensitivity testing platform validated to identify changes in drug responses caused by single mutations. At this point we cannot validate the drug sensitivity testing platform to identify changes in drug responses caused by single mutations.

Major Task 3/Subtask 1: Creating CRISPR-Cas9n – target mutation modified cell lines. (15% of planned completed)

Due to problems in getting our intended *PIK3CA* mutated control cell lines established using CRISPR-Cas9, we decided to move on to our patient-derived truncal clonal mutations. We chose to start with *ASNA1* c.224C>G_p.P75R mutation as based on in-house RNA-sequencing results this gene is expressed in prostate cancer cell lines as well as in RWPE-1 cell line. We chose to introduce also this mutation to RWPE-1 normal immortalized prostate epithelial cells to avoid as much as possible any accumulated genomic aberrations that are found prostate cancer cell lines.

ASNA1 sgRNA and HDR repair template design, sgRNA cloning and sgRNA testing were performed as described above for *PIK3CA*. These guides had CRISPR efficiencies of 50-60% when tested in 293T cells (Figure 2). We have just transfected these guides into RWPE-1 cells and are in the process of growing clonal cell lines.

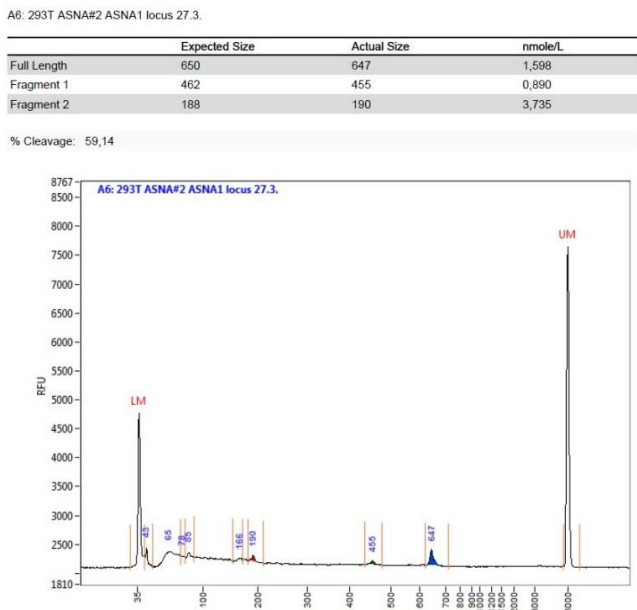


Figure 2. Representative image of SURVEYOR assay analysis using CRISPR Discovery Gel Kit with Fragment Analyzer (Advanced Analytical). 293T cells transfected with *ASNA1* targeting sgRNA #2. Three peaks were detected (full-size amplicon and cleavage fragments), and the % cleavage calculated from molar concentration of each peak represents to the CRISPR efficiency of given sgRNA.

When we have clonal *ASNA1* mutated cell line(s) established, we will have the drug sensitivity testing done in Karolinska Institute in collaboration with Dr. Päivi Östling. Once we have *ASNA1* ready as a clonal cell line, we will proceed to the next patient-derived mutation, namely *ABCC4* c.2672G>T_p.R891L, also highly expressed in some prostate cancer cell lines. We decided not to pursue

more than one mutation/gene at a time, since cell culturing at the clonal establishment step tends to be a bottle neck. As a deviation from the original plan, we decided not to create *PIK3CG* mutation carrying prostate cell lines, as the expression of *PIK3CG* is very low in prostate cancer cell lines as it was in the patient from whom it was first discovered, making it less likely to be druggable.

4. IMPACT

With the current progress of the project, there is nothing to report in terms of impact.

5. CHANGES/PROBLEMS

As stated above in research-specific tasks (Major Task 1/Subtask 2), we decided not to use the Cas9n-plasmid but instead the plasmid expressing wtCas9. This was because sgRNA design turned out to be too challenging when two high-quality, PAM-silently mutable guides were needed close to the wanted mutation site/cut site.

Also, as stated above in research-specific tasks (Major Task 1/Subtask 3), we faced a lot of difficulties in getting CRISPR working efficiently in RWPE-1 cells at various *PIK3CA* mutation loci. We suspected this being, at least for some part, due to the inaccessible chromatin-status in these cells that endogenously express very low levels of *PIK3CA*. Our transfection controls using GFP-expressing plasmid showed nice transfection efficiencies (up to 90%), so we excluded failed CRISPR-Cas9/sgRNA delivery from the causes of poor CRISPR efficiency. Additionally, in another cell line the chosen guides showed to have CRISPR efficiencies varying between 20-60%, suggesting that the observed maximum 15% CRISPR efficiency in RWPE-1 cells had other factors affecting the efficiency. Thus, we decided to move on creating cell lines with our patient-derived clonal truncal mutations. Work with *ASNA1* c.224C>G_p.P75R mutation has now been started and we have functional guides and we are currently in the process of creating clonal cell lines in RWPE-1 cells. We are also researching other options for control cell lines, i.e. genes that are expressed in normal prostate/prostate cancer cells and have known single nucleotide mutations that can be targeted with existing drugs.

6. PRODUCTS

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Hanna Rauhala
Project role:	PI
Researcher identifier:	0000-0001-7796-9117
Nearest person month worked:	12
Contribution to Project:	Dr. Rauhala has planned, executed and analyzed all the experiments performed so far.
Funding support:	DoD

Name:	G. Steven Bova
Project role:	Mentor
Researcher identifier:	0000-0003-1639-3104
Nearest person month worked:	1
Contribution to Project:	Dr. Bova has contributed in planning the experiments as well as helped rethink the experimental plan where needed.
Funding support:	University of Tampere

Name:	Päivi Östling
Project role:	Co-investigator
Researcher identifier:	0000-0001-5501-466X
Nearest person month worked:	0
Contribution to Project:	Dr. Östling has not contributed to the project during the first year as drug sensitivity testings have not been started yet.
Funding support:	SciLife, Karolinska Institute

Name:	Matti Nykter
Project role:	Co-investigator
Researcher identifier:	0000-0001-6956-2843
Nearest person month worked:	0
Contribution to Project:	Dr. Nykter has not contributed to the project during the first year as drug sensitivity testing analyses have not been started yet.
Funding support:	University of Tampere

No changes in personnel or organizations.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

Nothing to report.