

Jury Member Report – Doctor of Philosophy thesis.

Name of Candidate: Andrey Krivoy

PhD Program: Life Sciences

Title of Thesis: Primed CRISPR-Cas adaptation in type I-E system of Escherichia coli: use of single-molecule and biochemical assays to verify models of the phenomenon at molecular level

Supervisor: Prof. Konstantin Severinov

Chair of PhD defense Jury: Prof. Konstantin Lukyanov

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Date of Thesis Defense: November 30, 2018

Name of the Reviewer: *Petr Sergiev*

I confirm the absence of any conflict of interest	Signature:  Date: 11-11-2018
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The purpose of this report is to obtain an independent review from the members of PhD defense Jury before the thesis defense. The members of PhD defense Jury are asked to submit signed copy of the report at least 30 days prior the thesis defense. The Reviewers are asked to bring a copy of the completed report to the thesis defense and to discuss the contents of each report with each other before the thesis defense.

If the reviewers have any queries about the thesis which they wish to raise in advance, please contact the Chair of the Jury.

Reviewer's Report

Reviewers report should contain the following items:

- Brief evaluation of the thesis quality and overall structure of the dissertation.
- The relevance of the topic of dissertation work to its actual content
- The relevance of the methods used in the dissertation
- The scientific significance of the results obtained and their compliance with the international level and current state of the art
- The relevance of the obtained results to applications (if applicable)
- The quality of publications

The summary of issues to be addressed before/during the thesis defense

The thesis "Primed CRISPR-Cas adaptation in type I-E system of Escherichia coli: use of single-molecule and biochemical assays to verify models of the phenomenon at molecular level" by Andrey Krivoy is devoted to the fundamental study of the CRISPR/Cas systems, particularly to the process of primed adaptation. This process was discovered in professor Severinov laboratory, where the project was conducted. The literature overview goes about CRISPR/Cas systems, their classification and mechanisms of action, including an interference and adaptation processes. Primed adaptation is triggered by an incomplete matching of protospacer to spacer sequences and manifested in reduced interference paralleled with high efficiency of new spacer acquisition. Surprisingly, while complete match of protospacer to spacer sequences trigger highly efficient interference, the new spacer acquisition is almost entirely suppressed. This reciprocal negative influence, documented in the in vivo system screamed for some sort of a molecular explanation. The major suggestions are a concept of specific adaptation-type conformational change and a concept of kinetically driven acquisition. The most needed for the resolution of this puzzle is in vitro experiments. These experiments were the basis of the thesis proposal.

Andrey used a magnetic tweezers approach to examine the kinetics of R-loop formation upon crRNA interaction with the target DNA and dissociation of this functional complex. Magnetic tweezers allows monitoring the distance between the miniature balls, which could be artificially rotated in the magnetic field and the surface. For the application of the system to this case the DNA target of the CRISPR system was attached to the ball and the surface, so that the distance between these objects are indicative for the R-loop formation and decay. Andrey measured the kinetics of R-loop formation/dissociation (where possible) for a number of the templates either completely or incompletely matching to the crRNA. The differences were catalogued and discussed. Unsurprisingly, the seed and PAM region mutants demonstrated the most influence on both on and off rates of R-loop formation. Unexpected results were obtained for the elongated crRNA, which formed the locked complexes of conventional lengths.

Additionally to the magnetic tweezers experiments, Andrey performed a number of in vitro binding assays, Cas3-assisted degradation assays as well as an attempt to reconstruct entire primed adaptation system in vitro. For completeness of the work, in vivo adaptation experiments were performed on exactly the same templates. The most interesting finding is the comparison of the WT (fully matching) template with T4G substitution. Mean R-loop formation times for these variants are equal and both could not dissociate after locking. Only if the length of complementary region was shortened from the distal end, which is prohibitive for locking, the difference in R-loop formation and dissociation kinetics became apparent. Surprisingly, while the T4G variant induces priming, the WT do not. This is evidence that priming is not correlated with binding and locking.

While an attempt to reconstruct entire primed adaptation system was made, it was not successful.

I think that Andrey Krivoy made an excellent work, at the top international level of the field. He has two publications in the international scientific journals, including one, in Nucleic Acids Research (impact factor 11.5) as the first author.

While I'm sure that the results are undoubtedly convincing, I would like rise a few points.

1. Dissociation curves shown at the Figure 9A seems biphasic to me. Is it just an impression or there is some explanation of this fact?
2. I think it might be too preliminary to regard the results of in vitro spacer acquisition experiments as negative. I would suggest using the PCR-based method for monitoring spacer acquisition, similar to the

in vivo assay.

3. I could not get a clear idea on why T4G mutant support acquisition, while the WT do not. The mutant T4G, except for the variant with additionally shorter complementary region, behaves absolutely identical to the WT in all in vitro assays, including degradation. To support kinetic model, perhaps one would expect slower binding or slower degradation?

These suggestions, for sure, do not question the overall validity of the conclusions. I'm perfectly sure that the thesis is an example of an excellent scientific work. Andrey Krivoy should without any doubts defend the thesis by means of a formal thesis defense.

Provisional Recommendation

I recommend that the candidate should defend the thesis by means of a formal thesis defense

I recommend that the candidate should defend the thesis by means of a formal thesis defense only after appropriate changes would be introduced in candidate's thesis according to the recommendations of the present report

The thesis is not acceptable and I recommend that the candidate be exempt from the formal thesis defense