CORONASENSOR – Report on lab activity (April-May 2020) on testing Prototype v0.1b

With reference to the section “Description of testing methods” at https://coronasensor.uniud.it/latest-prototype the laboratory work has concerned points 3 (MNAzyme catalyzed reaction, including target recognition and signal amplification) and 5 (visualization of the results); other points were not investigated (but note that point 4 will not be required, as discussed below). The laboratory work has started with the setting up of the visualization stage, as the H1 and H2 oligos took more than one month to be available for testing, due to synthesis and logistic hindrances.
The plasmonic detection method has been tested and although it appears to be highly sensitive and easy to score with the naked eye, it has three important drawbacks: (i) nanoparticle aggregation is very sensitive to contaminant traces, hence not suitable for the household environment; (ii) it is also very sensitive to the presence of surfactants, which must be included in the lysis buffer and are difficult to remove before final visualization; and, most important (iii) the method is dependent on the amount of hydrogen peroxide left in the reaction well after the action of the peroxidase activity, not on the amount of peroxidase activity itself: hence the correct amount of hydrogen peroxide must be added very precisely at the beginning of the visualization step, and this is hardly compatible with the household compatible setup.
Visualization of the reaction products may be carried out with non-plasmonic, more conventional technology, i.e. using a chromogenic substrate. Several substrates for peroxidase exist, they perform variably with the HRP-mimiking activity of the G-quadruplex DNAzyme (from now on: Dz) used here and hence were tested extensively before proceeding with the work. ABTS is reported to be the most sensitive but it is not well suited for readout with the naked eye. We selected TMB, although the background is relatively high as it is oxidized to a certain degree by hemin alone, even in absence of Dz. The amount of hemin needed therefore optimization (fig.1), and the 5 uM allowed the best resolution in 30-40 minutes development times. With longer development times lower amount of hemin may be preferable for higher sensitivity. The standard procedure requires that hemin is pre-incubated before development to promote the formation of the G-quadruplex. In our tests 20 minutes pre-incubation was found sufficient. Further testing showed that pre-incubation may be skipped including hemin in the MNAzyme reaction, with no or modest loss of sensitivity. Although the TMB development reaction requires a more acidic pH, the solution for TMB visualization can be added directly to the MNAzyme reaction with some limited loss of sensitivity, making the chromatography step no longer necessary; hence the inclusion of hemin in the MNAzyme reaction allows to skip point 4 in the “Description of testing methods”, with enormous simplification of the final device.

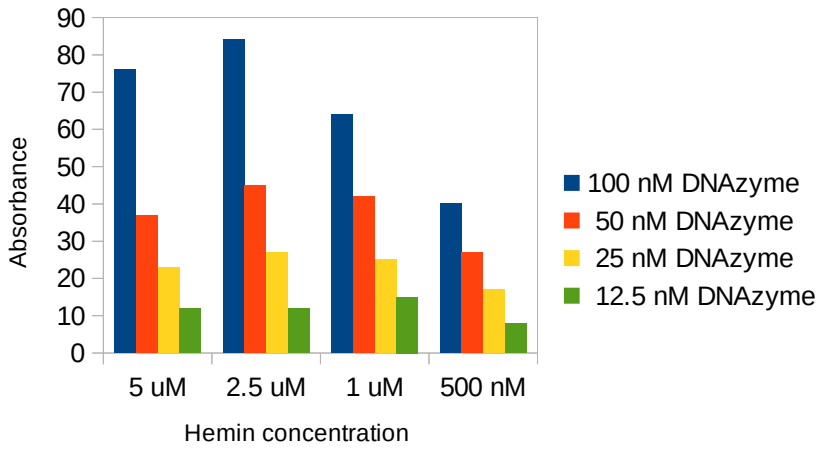

Figure 1: Relative absorbance (arbitrary units) given by reactions with different amount of hemin containing only Dz in different concentrations.

TMB visualization is not highly sensitive and it has been difficult to detect Dz at a concentrations below 10 nM. This should in theory not be a problem, since the MNAzyme reaction appears to be an end-point chain reaction (each cut by the MNAzyme complex ultimately produces a Dz and a new MNAzyme complex, exploiting linker) that is expected to proceed until either the linker of the hairpins are consumed. The reaction starts with 100 nM linker and hairpins, hence >100 nM Dz should be produced at end point. However, when tested in the lab when finally H1 and H2 arrived, the reactions carried out according to the methods did not produce the expected results for the following reasons:

1. The no-template control produced a relatively strong signal. In other words, Dz were produced in the negative control without the virus nucleic acids. The problem was due to the H1 and H2 oligos that had a residual peroxidase activity when hairpins formation was attempted using the program 37 (H1H2HEAT) in our "blue" PCR machine (fig. 2). More careful production of the hairpins (using the old style protocol, i.e. 5 minutes boiling then cooling to room temp overnight in a 500ml beaker) reduced but did not suppressed the problem. After emailing the authors of the original paper and following carefully the original protocol (boil 5 minutes then cool by 1°C/min, which required extensive manual programming of the PCR machine in the micologia lab), there was some further improvement but the residual activity of 50 nM H1 hairpin still had an absorbance similar to 10 or 20 nM Dz. Oligos need re-design with better masking of Dz in the hairpin; in the present design only 3 bases of Dz are involved in the hairpin and this does not seem to be enough, I our hands, to suppress G-quadruplex formation completely.

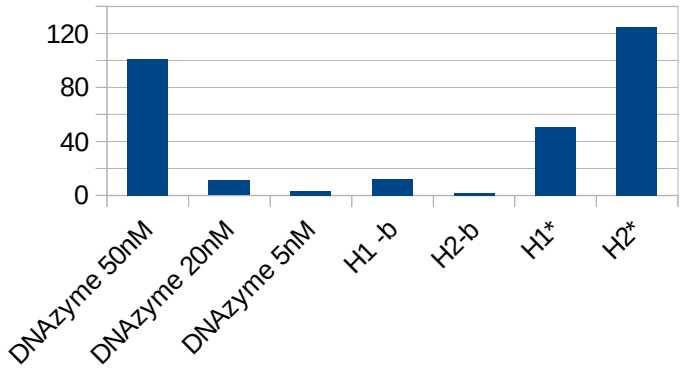

*Figure 2: Absorbance of 50nM H1 and H2 alone. The series * was done with program 37, the series "-b" with 1°C/min cooling*

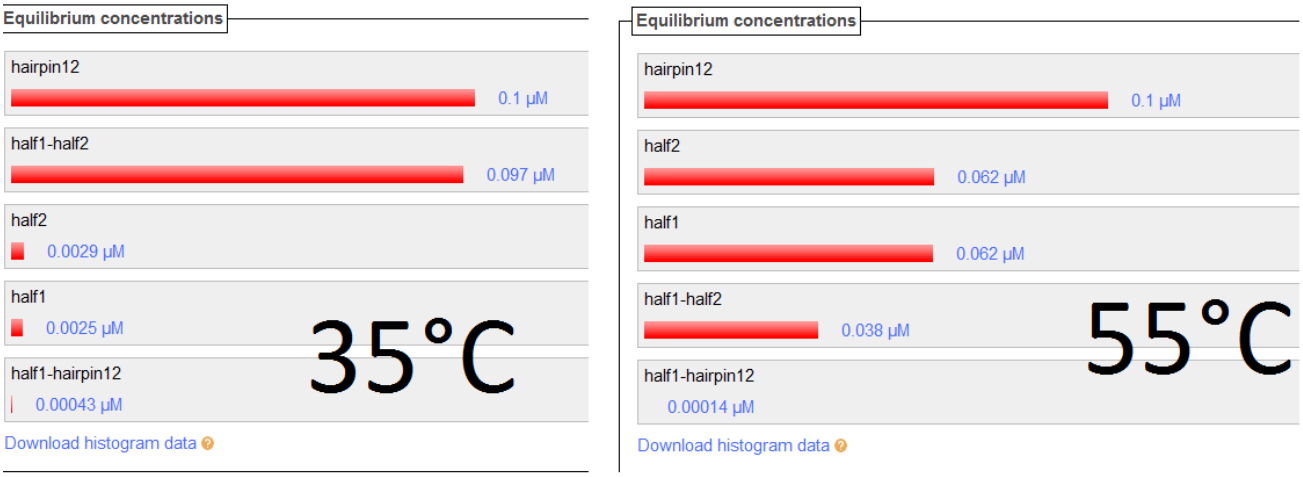

Figure 3: Nupack simulation of melting profile of the uncut hairpin (harpin12) and of the two halves resulting from MNAzyme cut.

2. The principle governing the reaction is that when the MNAzyme cuts the hairpin the two halves of the hairpin get separated because are thermodynamically unstable. Since the hairpin is closed with a 10 base pairs, it is not obvious that the two halves will get apart substantially at 37°C. Indeed according to NUPACK simulation the digested halves need higher temperatures to melt, as 97% of the stem of the

cut hairpin is still double stranded (half1-half2) at 35°C, while at 55°C most of the stem is melt and 62% of half1 and half2 exists as single stranded DNA (fig.3).

Carrying out the reaction at 55°C indeed produced some signal over the background as a result. The results displayed in fig.4 (exp20) are rather good (apart the sensitivity, since tq=100nM), but they could not be repeated in the last two experiments, when th e results were rather inconsistent. The temperature is relatively high for efficient annealing of the linker, therefore one possibility is that the chain reaction does not start or does not start consistently. Moreover the reaction could not be extended to more than 2 hours because the strength of the signal of the Dz Control (C+) decreases with incubation time, meaning that there is some degradation going on. This point needs further investigation to ascertain whether the short life is due to chemical degradation (hydrolitic) or enzymatic (contamination). Natural degradation at high temperature of DNA oligonucleotides containing a ribobase (as H1 and H2 are) has been reported by other and may be the reason for inconsistency of the results. In general, it seems not very appropriate to include a reaction that relies on a relatively high concentration of Mg++ in a diagnostic test to be carried out in the household, where the risk of contamination with Mg-dependent nucleases is assumingly high.

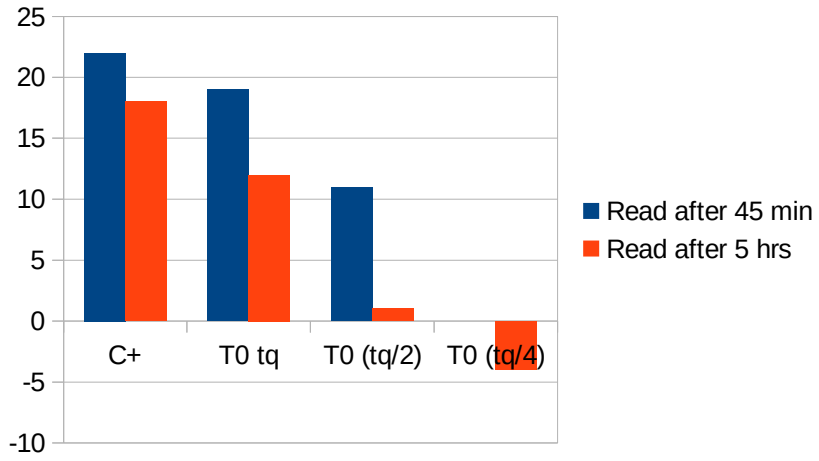

Figure 4: Relative absorbance value resulting from the reaction carried out at 55°C; the no target DNA control was used as blank

In conclusion, a substantial redesign of the oligonucleotides is needed to (i) eliminate the background signal due to incomplete inhibition of the Dz before MNAzyme cut and (ii) allow the chain reaction to proceed with efficient separation of hairpins halves after the cut and efficient annealing with linker to re-build the MNAzyme complex. Once the above issues would have been solved, then the definition of the LOD (limit of detection) of the procedure could be investigated. However, the evidence of several critical points in the work carried out so far (the stability of the hairpins, the limited difference in the melting patterns of the stems in cut vs. uncut hairpins, the sensitivity to nuclease degradation) do not allow much optimism in the possibility that the necessary high sensitivity could be actually reached. In our opinion, the way to proceed with this project must consider the inclusion of a different amplification method, as the method proposed by Xie et al. (2020, cited in the webpage) is not suitable for the kind of household test that we envisaged. According to the work done, the visualization using Dz and TMB can be successfully set for the household format; the MNAzyme recognition of the target is a powerful mean to avoid the use of antibodies and enzymes in the diagnostic test, although it needs further investigations; that the amplification step must be completely re-considered or substituted. A paper reporting the use of MNAzyme for recognition and Dz for visualization in the detection of the Zika virus has been recenly published (doi:10.1016/j.snb.2018.11.147), it uses NASBA as the amplification method. NASBA is an enzyme-based amplification methods and therefore would not fit in the enzyme free strategy of the coronasensor project.

Udine, June 8th, 2020