The following is a point-by-point response to the comments from peer reviewer no 1, the responses are written in italics.

- DBS method should be described more in detail
    - More details are now added to the Material and Method section.
- The selection and inclusion criteria of the population or subjects studied need further explanation
    - *Denmark is a low prevalence country for all three blood borne infections. To be able to perform a reliable evaluation of the method, we selected to evaluate the method in*
        1. *Known risk population (PWID) with a high HCV-prevalence of 30-40%, all PWID or prisoners with known HCV visiting the Drug treatment Center or incarcerated in the study period was invited for participation*
        2. *Known HBV or HIV infected patients followed in our outpatient clinics, all patients visiting the Outpatient Clinic in the study period were invited for participation*
    - *This has been specified in the Material and Method section*

- Most subjects seem to be known positive patients, and thus appear to be somewhat at variance with the intended claim to test difficult to reach populations not yet tested. The justification for the underlying prevalence of 30% has to be explained
    - *To be able to investigate the <u>real-life performance</u> of the test, we had to include known infected individuals. Part of the study population was hard to reach drug users attending drug treatment centers which also gave valuable information about the acceptance of the test in this population*
    - *As for the expected prevalence of 30%, this number is based on previous studies in Denmark showing a prevalence of HCV around 30-40%, declining over the last years due to change in drug intake habits (i.v. drug use is declining, and smoking increasing)*
    - *This has been clarified in the M & M section*

- Another aspect is the differentiation and assessment against detection of primary infections, which particularly play a role in risk groups
    - *As for standard venous blood sample testing there is a risk of testing in the window period and missing a newly infected individual, however using anti-HCV, anti-HIV and HBsAg we have shown that for these markers the sensitivity is very high DETECTING the infected individuals, which enables further follow-up where differentiation of primary or chronic infection can be made.*

- The overall classification of the sensitivity, for instance with respect to rapid point of care or self testing which may have similar use, may be extended
    - *We agree that validated point-of-care test for HIV and HCV do exist, but to our knowledge there is no formally validated POC for HBV neither in Western countries nor by the CDC in USA. The sensitivity of the POC has been described as slightly lower than standard serological tests, but for some tests comparable to Dried blood Spot (DBS) testing.*
    - *However one advantage of DBS is that chronic infection with HCV, HBV or HIV can be sampled at once, in comparison to POC-tests which are selective for one infection.*

    - *With POC test an answer is available within less than an hour. However literature has shown that even though different rapid POC assays for HBsAg are commercially available, some challenges still exist due to a wide range of diagnostic accuracy, different HBV genotypes (A–J) and HBV variants carrying amino acid substitutions which all combined increases the risk of escaping detection of HBV infection.*

    - *We wished to investigate the real-life performance of a DBS which enables examination for all three infections at once, and has been shown having high sensitivity using a modern high throughput analytic platform, enabling large screening campaigns in risk groups.*
- Page 5, last paragraph: Patient selection should be described more in detail. What were the criteria for selection of the subjects?
    - *Patients had to have known HBV, HCV or HIV*
    - *Patients had to attend either the Outreach clinic in the Drug treatment center or the Outpatient clinic at the Hospital to be eligible for inclusion if interested. Patients were informed about the study at already planned visits, if willing to participate they were included with due consideration time.*
    - *Clarification of selection and inclusion criteria has been added to the section; Study design*

- ? Page 6, to the M&M section: Given the subsequent discussion on the sample volume it should be described in more detail how the sample volume was determined
    - *We have added a description for this in the M&M section. Before the real-life collection of the samples was conducted, a pilot study was performed, where exact volumes of whole blood were added to the Whatman paper. Based on the pilot study approximately 75 microliter of whole blood was the amount needed to fill out a single spot on the Whatman filter paper. In the real-life study the intention was to fill out each of the 5 spots guided by visual appearance, and thereby approximating a volume of 75 microliter in each spot.*
- The DBS-processing method is not described in sufficient detail. How exactly the factor come about?
    - *We assume that the amount of plasma in 75 microliter whole blood with a hematocrit of 40 would be 45 microliter plasma. The dried blood spot was eluted in 1000 microliter buffer and therefore we estimated the dilution factor to be 1:23*

- Page 7, to sample size and power estimation: How is the expected prevalence of 33% based on
    - *The expected prevalence is based on the following calculation; Including 100 known infected patients with each infection (100 HCV, 100 HBV and 100 HIV, gives a prevalence of 33% of each infection (100/300)*
    - *Clarification has been added to the manuscript*

- Page 9, to Hepatitis B: From <175 IU/ml to the test cutoff of about <0.1 IU / ml there is a very broad range. In this area, one would actually expect a reliable positive test result. Otherwise the question would arise whether weak positives can be detected at all. Can this figure be shown more limited?

    We agree this is a broad range but lowering the cut-off would risk produce false negative results in our study, hence being pragmatic we chose <175IU/ml based on our results. However one has to bear in mind that due to the low prevalence of HBV in Denmark we had

to include treated HBV patients with low or undetectable HBVDNA, and this will not be the case when using DBS as a screening method among patients with unknown HBV status

- Page 10 1st paragraph regarding: "CMIA median 6.19, range 1.1-10.1". One can imagine that values only slightly >1 s/co in Architect Anti-HBc as shown here can be false negative after dilution by the elution of DBS. This should be further explained.
    - *Yes we agree this is a possible pitfall, and one of the limitations of the DBS method in real-life. HBsAg is used as the marker for HBV-infection (acute or chronic) and anti-HBc is not essential at the screening point in time in the determination of if the patient is infected with HBV or not. However screening positive individuals should have a full serological panel done, and hence it is important to know the limitations of the DBS method.*

- Also for Fig. 2 the HBV algorithmus does not include clarification of the aHBc results to exclude false positives?
    - *Due to the limitations of the anti-HBc performance we decided not to include the anti-HBc results in the figure as no further information is added.*

- Page 10, 3rd paragraph: Anti-HBs was positive in plasma (1.08-9.44 mIU/ml) but negative in DBS (0-0.3 mIU/ml). According to the Architect test interpretation criteria, only aHBs values ≥ 10.00 mIU/mL are considered reactive. Therefore, this appears to be only seemingly a discrepancy.
    - *This is correct, however in this study the purpose was to investigate the sensitivity of the DBS-test, and changing the cut-off value to 10mIU/ml does not change the results as the discrepancy remains lower due to dilution factor, we investigated if using the calculated cutoff by Ross et al would improve the sensitivity it did to some degree (42% ->53%), and this is included in the discussion.*

- Page 10, 4th paragraph: 79 where plasma was positive (median 9.9 mIU/ml, range 1-75) and DBS negative (median 0.01 mIU/ml, range 0-0.93). The question arises whether this is not a value range, which is negative after the dilution in the eluent DBS. This should be explained further.
    - *We agree, as for HBV-infected, the value range found among non-HBV infected is most probably due to the dilution factor, however it remains a limitation when using DBS and one should be aware of this. This has been added to the discussion section*

- Page 11, last paragraph to sentence: "The low sensitivity, for the serological markers; anti-HBs and anti-HBc in DBS versus plasma, found in our study is in contrast to recent studies using automated platforms". This may be an combined effect of different starting titers of the sample and different dilution factors by elution. Can the discussion be extended to?
    - *We agree and this has been added to the Discussion.*

- Page 12, 1st paragraph to sentence: "We speculate if an indicator for the amount of blood collected on the paper could be developed to insure that enough blood is present for sampling (e.g. weight, haemoglobin etc). This would also us allow to calculate a quantitative antiHBs /ml of serum from DBS, to be used in outreach vaccination trials." The increase of the sample input volume, e.g. as mentioned above from approximately 75 ul to 100 ul seems to be to be rather small compared

against the dilution effect of the elution (factor 23) and differences in this dilution factor. This should be explained more.

- *We agree; however the turning point is not the difference from 75 to 100 microliter, but the uncertain amounts of blood applied in real-life settings. Awareness should be taken and additional methods to approximate the added volume of blood are needed, possible methods are suggested in the discussion.*

- Page 13, 1st paragraph to sentence: “We therefore suggest that treatment na?ve patients that are anti-HCV positive/HCVRNA negative in DBS screening (suggesting past HCV infection) should have their status confirmed by a subsequent venous blood sample.” This may not be the only constellation, since single negative HCV-RNA results may not determine the infection status due
    - *We are aware of that this is not the only constellation; however this method is suggested as a screening method to be repeated yearly in risk population to identify chronic infections. This has been added to the Conclusion.*