Author's response to reviews

Title: Dual color chromogenic in situ hybridization for determination of HER2 status in breast cancer: a large comparative study to current state of the art fluorescence in situ hybridization

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Author's response to reviews: see over
Dear Natalie Pafitis, Senior Executive Editor

Thank you for the meaningful and constructive comments from the reviewers. We have introduced the suggested changes with minor exceptions. Below we give an overview of the changes to the manuscript and we address the points raised by the reviewers point-by-point. Overall we think that manuscript has improved and we are looking forward to receive your response.

On behalf of all authors,
Yours sincerely,
Jens Mollerup
Reviewer: Benjamin Kipp
Minor Essential Revisions

1. Please elaborate on how the specimens were evaluated. Were the specimens evaluated in blinded fashion? Did the same person evaluate all specimens? Who evaluated the specimens (technologist, pathologist, etc)? Most importantly, did the person resulting the CISH assay already know the results of the other assay(s)? How were the reviewers blinded to the different test results to eliminate potential bias?

Author response: We agree that this could be clearer. Therefore, we have introduced a paragraph in the Methods section that answer these questions. This paragraph describes how specimens were evaluated by three different technologists and two pathologists and also that knowledge on test results were not shared between observers during the study.

Paragraph inserted line 5 from bottom of page 4:
“Evaluation of specimens were performed by three different technologists for the three ISH tests and subsequently reviewed by the pathologists. One pathologist reviewed test results obtained with PathVysion HER-2 DNA Probe Kit and HER2 CISH pharmDx™ Kit with several months in between and another pathologist reviewed test results obtained with HER2 FISH pharmDx™ Kit. Knowledge of test results was not shared between technologists or between pathologists.”

2. Please elaborate on the discordant cases. Were the discordant cases most often in the equivocal category and close to assay cut-offs? Were the discordant results due to tumor heterogeneity or possibly interpretation error? Also, among specimens that were discordant, what were the results of the third test? Did it often agree with the CISH assay or the other assay? This information will allow the reader to better understand the rare discordances.

Author response: We also think that information regarding discordant cases could be interesting to the reader. Therefore, we have inserted two paragraphs in the Results section page eight that give information regarding the IHC score of these cases as well as the proximity to the HER2/CEN-17 ratio cut off (see below). Whether discordant results were due to heterogeneity or interpretation error or something else is very difficult to say. Generally discordant cases are close to the assay cut off. Due to variance between observers and sections of the same block it is common to get ratios that may be on either side of the assay cut off. The “third test” was either PathVysion FISH (for comparison between HER2 CISH and HER2 FISH pharmDx™) or it was HER2 FISH pharmDx™ for comparison between HER2 CISH pharmDx™ and PathVysion FISH) and there was no tendency as to whether agreement was better between the “third test” and HER2 CISH pharmDx™. However, three discordant cases were a result of the appearance of CEN-17 clusters, and a notice regarding the appearance of clusters of blue signals has been inserted in the manuscript Result section as can be seen in the second paragraph inserted below.

First paragraph inserted from line 10 page 8:
“Three of the six discordant cases for the comparison between HER2 CISH and HER2 FISH (Table 3) were HercepTest™ IHC 2+ equivocal cases, and the remaining three were 0, 1+ and 3+, respectively. The discordant cases had a HER2/CEN-17 ratio very close to or within the borderline area defined from 1.8 and 2.2 for at least one of the three methods performed.”
Second paragraph inserted line 5 from bottom page 8:

“Five of the eight discordant cases for the comparison between HER2 CISH and PathVysion FISH (Table 4) were HercepTest™ IHC 2+ equivocal cases, and the remaining three were 3+. For the three IHC 3+ discordant cases cluster amplification of blue (CEN-17) signals was observed that covered red (HER2) signals or made them difficult to see. In these cases the blue signals in the normal cells surrounding the tumor cells were clear and distinct and cases could therefore pass the quality control. Therefore, in cases with cluster amplification of blue signals additional caution should be taken during interpretation and results from other test methods such as IHC or FISH should be included before a final HER2 status is given.”

3. It appears that the CISH pharmDX kit has been recently approved by the FDA. This should be updated in the manuscript.

Author response: This is correct and we have modified the text a few places to accommodate this new information.

In the Abstract section the Conclusion paragraph has been slightly rephrased from:

“The concordance between results obtained using the FDA approval pending HER2 CISH pharmDx™ Kit with today’s FDA approved FISH techniques for HER2 gene status determination indicate that the HER2 CISH pharmDx™ Kit is a reliable chromogenic alternative to approved fluorescence-based methods.”

To:

“The concordance between results obtained using the recently FDA approved HER2 CISH pharmDx™ Kit with previously FDA approved FISH techniques for HER2 gene status determination indicate that the HER2 CISH pharmDx™ Kit is a reliable chromogenic alternative to fluorescence-based methods.”

Line 9 from the bottom of page 9, where “FDA approved” has been taken out was changed from:

“The plot of the ratio data revealed a good correlation between the HER2 CISH ratio and the ratios obtained by two FDA approved HER2 FISH assays.”

To:

“The plot of the ratio data revealed a good correlation between the HER2 CISH ratio and the ratios obtained by the two HER2 FISH assays.”

Line 4 page 11 where “FDA approved” has been taken out was changed from:

“For overall agreement the lower 95% confidence intervals were at or above 96% in the two comparisons, further stressing the reliability of the HER2 CISH pharmDx™ in this comparison to the two FDA approved FISH analysis methods.”

To:

“For overall agreement the lower 95% confidence interval limits were at or above 96% in the two comparisons, further stressing the reliability of the HER2 CISH pharmDx™ in this comparison to the two FISH analysis methods.”

The final Conclusion bottom of page 12 has been slightly rephrased from:

“From this study based on HER2 CISH and FISH data from 365 different primary breast cancer specimens it is confirmed that the HER2 CISH pharmDx™ Kit is a reliable chromogenic alternative (HER2 CISH pharmDx™ Kit FDA approval pending) to today’s FDA approved FISH techniques for HER2 gene status determination in FFPE breast carcinoma specimens.”

To:

“From this study based on HER2 CISH and FISH data from 365 different primary breast cancer specimens it is
confirmed that the FDA approved HER2 CISH pharmDx™ Kit is a reliable chromogenic alternative to today’s FDA approved FISH techniques for HER2 gene status determination in FFPE breast carcinoma specimens.”

Discretionary Revisions
1. A Table comparing the HER2 FISH and PathVysion results (similar to Tables 3 and 4) may be interesting to the reader.

Author response: We recognize that this might be interesting for some readers, however, data on this comparison has already been published and is also available in the package insert for the HER2 FISH product and we therefore do not bring this comparison.

Reviewer: Zoltan Szollosi

Minor Essential Revisions
1. Although not TMA slides were used (and therefore not only few slides were hybridized), I would like to see information of the success rate of FISH vs. chromogenic hybridisation (=how many samples were repeated; how many were excluded due to technical failure)

Author response: We agree that information regarding success rates could be interesting for the reader. Therefore, we have inserted a separate paragraph “Success rates” in the Result section (see below) shortly describing the success rates for the three assays.

Paragraph inserted from line 6 page 9:

“Success rates

Final success rates were determined after allowing for two staining runs. Of the 13 cases with a missing HER2 CISH test result (Table 2) the second staining run was not performed in four cases and, therefore, the success rate for HER2 CISH was 97.5 percent (352/361*100). The success rates for HER2 FISH and PathVysion FISH were 98.4 and 98.9 percent, respectively.”

2. The authors did not mention the importance of the definition of polysome in HER2 diagnostics. The whole issue of polysomy and whether CEP17 probes can actually define polysomy has been called into question by Yeh et al. Mod Pathol. 2009 Sep;22(9):1169-75. Therefore, the authors should also report on the accuracy of each system in defining HER2 amplification based on a single probe as described in the ASCO/CAP guidelines.

Author response: We also think that the definition of polysomy in HER2 diagnostics is an important topic. The question whether CEN-17 enumeration truly describes polysomy has indeed been questioned in recent publications, and will most likely be investigated further in the future. We do not think that the data presented in this manuscript shed further light on the definition of polysomy, therefore, we refrain from discussing this topic. The predictive and prognostic data that links the HER2 CISH assay to its intended use is not based on the alternative definition of amplification based on a single probe that has been put forward by the ASCO/CAP. Therefore, we do not think it is appropriate to include concordance data based on HER2 signals only. Also it is worth noticing that in a recent Advance Access Communication by Perez et al. in J Natl Cancer Inst (December 2, 2011) there is very strong support for the FDA guideline since the use of the ASCO/CAP alternative guideline may defer a small group of women from potentially life-saving trastuzumab treatment. To emphasize that the HER2 CISH assay is based on the FDA guideline this has been stipulated in the Methods section in the paragraph “HER2 Status”.

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The paragraph line 9 page 6 has been changed from:
“For CISH and FISH methods HER2/CEN-17 ratios obtained were translated to a HER2 gene status of amplified when the HER2/CEN-17 ratio was higher than or equal to 2.0 or non-amplified when the HER2/CEN-17 ratio was below 2.0.”
To:
“In accordance with FDA approved guidelines for determination of HER2 status HER2/CEN-17 ratios obtained by CISH and FISH were translated to a HER2 gene status of amplified when the HER2/CEN-17 ratio was higher than or equal to 2.0 or non-amplified when the HER2/CEN-17 ratio was below 2.0.”

3. The images should be re-edited.
Author response: We have looked through the figures in the manuscript and could not identify any problem. Figures have been uploaded in the desired resolution.