Reviewer’s report

Title: A simple HyBeacon probe system enabling high-throughput genotyping of multiple single nucleotide polymorphisms closely positioned in the ovine PRNP gene

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Reviewer: Bertram Brenig

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Comments on “A simple HyBeacon probe system enabling high-throughput genotyping of multiple single nucleotide polymorphisms closely positioned in the ovine PRNP gene” by French et al.

The authors describe another probe-based ovPRNP genotyping assay. The overall novelty is limited and the paper lacks of several severe problems. This might be due to a limited understanding of the methodology and reading of literature in the field of fluorescence probe based genotyping.

Major Points
1) The use of Trade names in the title of a scientific publication should be limited to cases where unavoidable. The herein called HyBeacons show the underlying problem perfectly. These are just simple linear fluorescence probes but have nothing in common with molecular beacons, but share all hybridization properties with other linear probes.
2) The background section in the abstract is as an effect of point 1 very misleading, since again, what is said is true for all probe based genotyping and therefore (also) for the so called HyBeacons.
3) The assay is poorly designed and therefore requires three or four LightTyper runs to come to a genotype. The respective literature is full of publications about Tm-multiplexing also for ovPRNP (e.g. ref. 23). The chance to design a smart assay has been failed completely. The inability of the technology to employ color multiplexing needs to be addressed. There is a rhodamine-derivative simple probe in development by one of the major companies in the field, but as long as unavailable, it’s a major drawback that needs discussion.
4) The authors state that the assay is inexpensive, but refuse to show any respective cost analyses, which would be needed in any case. The multiple steps, such as 3 (4) PCRs followed by 3 (4) LT runs exporting the data import to Excel (BTW: ™ is missing) calculate ratio etc. etc. already show the enormous technician time needed and in turn the cost-effectiveness must be doubted.
5) The section about complex genotypes is a misunderstanding of the underlying cause, which is well known for ruminants (sheep, cattle etc.). This is a blood chimerism and only should have one consequence: if seen, genotyping from tissue is required: the effect is gone and the right genotype can be defined. This may be mentioned in one half-sentence, since most laboratories have switched to ear-tag tissue genotyping. Even though this has somehow managed to enter the literature, a frequency of 1 in thousand real germ line (tissue) chimerism in a mammal species is unconceivable to begin with and having tissue genotypes as one by one comparison would have been the only information worthwhile to be published in this context helping to get rid of this fallacy.
6) None of the figures is needed for the understanding of the manuscript. These are likely to be the nos. 956 to 962 melting curves in the literature.

Minor points (by page, not in order of significance)
1) “HyBeacon” and “high-throughput” are unacceptable for the title, in case of the first word for reasons laid out above. For the latter, the authors do not show any time/throughput calculations in the entire manuscript. Abstract same!
2) The function of the negative first derivative is wrong (pg. 9).
3) Terms like “majority” and “small number” are meaningless, starting on pg. 9 this goes throughout the manuscript, where any number can be found like 85% on pg.18 (direct) and 98% (same page for “combined” – how combined?). What is the reader thought to believe? Some reasonable statistics would help to clarify this confusion.
4) The 5°C difference (pg. 10) is not congruent with table 2, where lower differences are claimed to be unambiguous. Although the ref. 18 is not the best in this context, what should the reader believe now?
5) The reader is very often left alone with statements, where a literature reference could help. As an example (pg 13): “This K allele is very uncommon in the UK flocks…..” Please go through the text and cite literature where needed.
6) 1,000 samples (pg.14) can only be true for the major genotypes; be precise here!
7) Hydrolysis probes (called TaqMan here – cf. point 9) and scorpion primers are two complete different animals (pg 16). Scorpion hybridization is only comparable to molecular beacons and are used in a non-destructive manner offering the capability of post-PCR analysis. This would be advantageous over linear probes, based on the underlying two-state melting, leading to an increase in delta-Tm (see Bonnet et al. PNAS 1999; 96: 6171).

8) Following point 4, there is no thought about the inherent risk of failure to detect yet unknown mutations. This discussion is superficial and full of misleading statements. Any mutation causing a slight destabilization of the probe binding would be undetectable (in particular the 141 probe is prone to this). Need to be rewritten after reading of some literature.

9) The discussion about 5-nitroindole and alike should include recent trends for masking of unwanted regions/bases in these assays, which are more cost effective.

10) Overall the wording should comply with scientific standards, lots of sloppy terms (lab slang) are used e.g. “Melt analysis” when probe dissociation analysis is done “Melting temperature analysis” would be acceptable also, since it is often used for it in the literature.

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, and I have assessed the statistics in my report.