Appendix 1:

Additional Review of Sequence Data for the Center for Food Safety

by

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Summary of crucial points: (1) Analysis of the EO-1α transgene insertion site of *AquAdvantage* Salmon is incomplete and therefore inadequate and (2) the company has failed to prove that the EO-1α transgene they have characterized is the sequence responsible for the growth phenotype of *AquAdvantage* Salmon.

1. Analysis of the EO-1α transgene insertion site of *AquAdvantage* Salmon is incomplete and therefore inadequate

Yaskowiak *et al*. (2006) have sequenced the EO-1α transgene and 1136bp of upstream flanking sequence and 730bp of downstream flanking sequence from AquAdvantage Salmon. However they have not identified and sequenced the original insertion site from wild-type Atlantic salmon. Therefore, nothing meaningful can be said about the extent of genomic damage at the insertion site and the fundamental question, *Has insertion of the EO-1α transgene disrupted or deleted any important functional sequences in AquAdvantage salmon?,* remains unanswered.

For example:

i. It is possible that the 35bp repeated sequence flanking the EO-1α transgene is not the sequence of the original insertion site in wild-type Atlantic salmon and that the 35bp repeat sequence was either inserted or amplified when the EO-1α transgene inserted into the genome.
ii. As the full extent of deletion and rearrangement at the site of transgene insertion is unknown, it is possible that one or more genes have been deleted or disrupted during transgene insertion.

2. The company has failed to prove that the EO-1α transgene they have characterized is the sequence responsible for the growth phenotype of *AquAdvantage* Salmon.

Yaskowiak *et al*. (2006) and the FDA assessment (p.16) claim, "*AquAdvantage* Salmon currently used for production contain a single well-characterized copy of the construct at the α-locus." However, their experiments are unable to rule out the possibility that one or more copies of the Chinook salmon growth hormone DNA are located at a site genetically linked to the EO-1α transgene. This is because their

Southern blot analysis was carried out using probes to a fragment of 5' regulatory sequence and 3' regulatory sequence and not to the Chinook salmon growth hormone coding sequence itself (Yaskowiak *et al.* (2006), Figure 1 and discussion p. 471). Consequently, it is not possible to determine whether additional intact and functional copies of the Chinook growth hormone coding sequence are present in the *AquAdvantage* genome, at a site genetically linked to the EO-1α transgene. For example, the growth hormone sequence might have integrated near an endogenous promoter and/or it might have integrated with part of the 5' sequence of the ocean pout promoter (as only part of the 5' sequence of the ocean pout promoter was used in Southern blot analysis). If they exist, such copies, and any other non-functional fragments of Chinook growth hormone DNA inserted into the *AquAdvantage* Salmon genome, would of course introduce their own genetic damage into the *AquAdvantage* genome.

Note: It is clear that the Yaskowiak *et al*. (2006) paper and the publicly available data fail to address whether there are additional copies of Chinook growth hormone sequence genetically linked to the EO-1α transgene. However, based on the statements contained in the VMAC assessment, it is impossible to determine whether additional data presented to the FDA rule out this possibility (p. 16, subheading: PCR amplification).

Additional points to note about the AquAdvantage assessment:

A. Regarding the sequence of the EO-1α transgene insert:

We concur that the data presented in Yaskowiak *et al*. (2006) suggest that the original Chinook growth hormone construct (opAFP-GHc2) became rearranged during insertion and the genomically integrated EO-1α transgene consists of the following: a truncated ocean pout antifreeze 5' promoter region (bp 1580-2193), the intact Chinook salmon growth hormone cDNA (bp 2194-2897), intact ocean pout antifreeze 3' termination region (2898-4061), fragments of pUC9 polylinker (25bp total) and pUC18 polylinker (20bp total) and a fragment of the ocean pout antifreeze promoter region (bp 1-1678), in that order.

Note:

i. 45bp of polylinker DNA from two plasmids is present in the EO-1α transgene.
ii. 98bp of the ocean pout antifreeze 5' promoter region (bp 1580-1678) is tandemly repeated in the EO-1α transgene, as this 98bp sequence is present at both the 5' and the 3' ends of the EO-1α transgene.

It is not clear whether any risk issues arise from these rearrangements.

B. Regarding the presence of additional pUC plasmid DNA in AquAdvantage Salmon:

Prior to microinjection into Salmon eggs, the original Chinook growth hormone construct (opAFP-GHc2) was released from its plasmid vector sequences by restriction enzyme digestion. However, the DNA microinjected into salmon eggs to produce *AquAdvantage* Salmon included this plasmid vector DNA as well as the original GH construct (opAFP-GHc2)(FDA assessment, p.15).

This leaves open the possibility that plasmid sequences are present in the AquAdvantage Salmon genome. The FDA assessment claims southern blot data have been presented showing no plasmid DNA is present in the *AquAdvantage* salmon genome (p. 15 under subheading: *i. Plasmid DNA*).

However, the only data available to the public, the Yaskowiak et al. (2006) paper, do not address the question of whether there is plasmid DNA present in the *AquAdvantage* Salmon genome. Therefore, from the public's point of view there is the possibility that the *AquAdvantage* Salmon genome contains additional pUC plasmid sequences.

C. Regarding the significance of repeat DNA:

This is not a topic that we have any expertise in, however it seems clear that, at least in human genome research, repeats are no longer considered 'junk' DNA. Repeats may have important functions in the regulation of gene expression and higher order genome structure and repeat DNA present in coding sequences can be involved in disease.

So, were the company to prove that the flanking repeat DNA was indeed the original insertion site in wild-type Atlantic salmon (something which they have not done, see 1. Above), the fact that a transgene has inserted into repeat DNA is no compelling reason to believe that no alterations have occurred to endogenous gene regulation or that no important function has been disrupted.

D. Possibly interesting references:

i. **This paper shows that (a) insertion sites in salmon can be properly analyzed and (b) transgene insertion in salmon can result in substantial deletion and rearrangement of endogenous DNA:** Uh *et al*. (2006) *Transgene constructs in coho salmon (Oncorhynchus kisutch) are repeated in a head-to-tail fashion and can be integrated adjacent to horizontally-transmitted parasite DNA. Transgenic Research* **15(6):** 711-727.

ii. A paper describing the importance of repeats in human DNA: *The biological effects of simple tandem repeats: Lessons from the repeat expansion diseases* *Genome Res.* 2008 18: 1011-1019, Karen Usdin,
PDF file: http://genome.cshlp.org/content/18/7/1011.full.pdf+html?sid=3c6d2fc9-6755-480e-9880-c7e1ad109709