To whom it may concern

We have been asked by GE Free New Zealand (in food and environment) to comment on the AgResearch, Ltd reports

- "Microbial characterisation of soils from offal pits",
- "Monitoring offal pits for puromycin resistance bacteria and the presence of genes encoding resistance to puromycin",
- "Monitoring offal pits for the presence of puromycin resistant bacteria and the presence of genes encoding resistance to puromycin",
- "Monitoring offal pits for the presence of puromycin resistant bacteria and the presence of genes encoding resistance to puromycin", and
- "Monitoring offal pits for puromycin resistance bacteria and the presence of genes encoding resistance to puromycin"

dated from November 2004 to December 2009. As part of our public service and specifically our formation mission, the Centre for Integrated Research in Biosafety has accepted this task.

In order to provide the best possible interpretation of the findings reported, we were hoping that you would be able to answer a few supplemental questions. In return, we will provide AgResearch, Ltd. with an advanced draft of our final report to allow AgResearch to register with us any differences in interpretation before the report is released.

<u>Follow up questions for AgResearch monitoring.</u>

How many bacteria were actually counted in determining cfu/g soil? What proportion of a gram of soil was actually plated?

Where resistant bacteria were characterised by PCR, did AgResearch assume that any amplified products were also the DNA sequences that had been detected by the probes in the preceding stages of characterisation? If so, what evidence did AgResearch have to make this association?

Where PCR was used to attempt to amplify resistance genes, were samples taken from environmental DNA extractions and mixed with positive controls to determine both the sensitivity of the reaction and absolute amplification limit?

By what criteria were control site locations chosen? Were these criteria verified as appropriate? If so, how?

How did AgResearch determine the number of bacteria to screen? Were these targets based on ecological modelling or empirical data to confirm that the power of the analysis was appropriate?

<u>Report-specific questions.</u>

How does AgResearch account for the shift in number of CFUs between 2004 and 2008, particularly in control samples? That is, the range of CFUs in 2004 was control: 10^3-10^6, pit: 10^4-10^7, in 2007 control: 10^7-10^8 (a 10-1000x increase), pit: 10^7-10^8 and in 2008 control: 10^6-10^7, pit: 10^7 per gram of soil.

The 2007 report says: "A total of 687 individual bacterial isolated randomly selected from TSA isolation plates, both containing puromycin and without, were probed using a fragment of the puromycin resistance encoding gene from pOL71. In the control soils, 16 of the bacteria probed positive, while in offal pit soil 8.9% probed positive (Table 2)." We could not find this data in Table 2. Could you please supply it? Please also put the results into a common unit (rather than report numbers for one observation and proportion for another).

The 2007 report says: "Using antibiotics in the medium, 27.9% of colonies probed positive when isolated on puromycin containing medium". We calculate 26.9%. Could you confirm your number and indicate how it was calculated?

Why is the kanamycin resistance not followed past 2004? If it was not to be followed, what was the rationale for selecting on plates with kanamycin, and then not selecting for kanamycin in later reports?

Why were the recommendations and observations of the 2004 report not followed up? [2004 report (emphasis added): "Similarly, probing for the nptII gene found a number of positive colonies, which is interesting as we have never before detected nptII in New Zealand bacteria. *This result needs to be further investigated.* In previous work, we have not detected the nptII gene in New Zealand soil bacteria and *it is interesting that numbers of nptII gene-positive colonies were higher in the offal pit than in control soil samples.*]

Attachment for the response to Professor Heinemann's Official Information Act request dated 20th April 2010.

1. ***How many bacteria were actually counted in determining cfu/g soil? What proportion of a gram of soil was actually plated?***

This information is not held by AgResearch and is therefore not possible for us to provide under the Official Information Act.

2. ***Where resistant bacteria were characterised by PCR, did AgResearch assume that any amplified products were also the DNA sequences that had been detected by the probes in the preceding stages of characterisation? If so, what evidence did AgResearch have to make this association?***

There were no assumptions. The PCR assay is designed to amplify the exact sequence only. Where possible, any amplification product produced were sequenced to determine its identity.

3. ***Where PCR was used to attempt to amplify resistance genes, were samples taken from environmental DNA extractions and mixed with positive controls to determine both the sensitivity of the reaction and absolute amplification limit?***

Our assay does not involve the amplification from environmental samples. Thus, the suggested controls are not relevant.
In our assay, we are using highly pure DNA and the amount of DNA is not limiting. The target sequence was spiked into bacterial DNA at a calculated ratio of one copy target sequence/ bacterial genome. At the DNA template concentration used in the assay, the above positive control results in strong amplification and highly visible product indicating that the assay has adequate sensitivity.

4. ***By what criteria were control site locations chosen? Were these criteria verified as appropriate? If so, how?***

Control site locations were chosen based on similar soil type; the soil types are known for all Ruakura sites, and similar farming activities; the control site is used as paddock and subject to cattle grazing.

5. ***How did AgResearch determine the number of bacteria to screen? Were these targets based on ecological modelling or empirical data to confirm that the power of the analysis was appropriate?***

Approximately 500 bacteria covering + puromycin plates and – puromycin plates were analysed by colony hybridisation. These cover both control and ACU soils.

6. ***How does AgResearch account for the shift in number of CFUs between 2004 and 2008, particularly in control samples? That is, the range of CFUs in 2004 was control: 10e3-10e6, pit: 10e4-10e7, in 2007 control: 10e7-10e8 (a 10-1000x increase), pit: 10e7-10e8 and in 2008 control: 10e6-10e7, pit: 10e7 per gram of soil.***

Bacterial populations in soil change very rapidly. This can be due to climatic effects (soil temperature, rainfall event) or something else like availability of nutrients, the extent and quality of plant cover etc. Therefore it is not unexpected that the bacterial

count shows some variation from year to year. The intension was to monitor for potential HGT under normal operational conditions.

7. ***The 2007 report says: "A total of 687 individual bacterial isolated randomly selected from TSA isolation plates, both containing puromycin and without, were probed using a fragment of the puromycin resistance encoding gene from pOL71. In the control soils, 16 of the bacteria probed positive, while in offal pit soil 8.9% probed positive (Table 2)." We could not find this data in Table 2. Could you please supply it? Please also put the results into a common unit (rather than report numbers for one observation and proportion for another).***

Unfortunately, the percentage sign was missing. The 16 refers to 16% which can be calculated from the data provided in Table 2 by dividing the total number of positives for all control samples (54) through the total of all probed colonies for all control samples (338), multiplied by 100.

8. ***The 2007 report says: "Using antibiotics in the medium, 27.9% of colonies probed positive when isolated on puromycin containing medium". We calculate 26.9%. Could you confirm your number and indicate how it was calculated?***

The correct number is 26.9%. The inclusion of 27.9% in the report must have been caused by a typo. The calculation is as follows:
(Total number of puromycin selected colonies probed showing a positive hybridisation signal / total number of puromycin selected colonies probed) x 100

9. ***Why is the kanamycin resistance not followed past 2004? If it was not to be followed, what was the rationale for selecting on plates with kanamycin in 2004, and then not selecting for kanamycin in later reports?***

Kanamycin was initially included out of scientific interest. Since it adds significant costs but is not directly related to the monitoring of HGT from the transgenic cattle activities, screening for kanamycin was not continued.

10. ***Why were the recommendations and observations of the 2004 report not followed up? [2004 report (emphasis added): "Similarly, probing for the nptII gene found a number of positive colonies, which is interesting as we have never before detected nptII in New Zealand bacteria. *This result needs to be further investigated.* In previous work, we have not detected the nptII gene in New Zealand soil bacteria and it is *interesting that numbers of nptII gene-positive colonies were higher in the offal pit than in control soil samples.*]***

As with many interesting observations that may warrant further investigation, it represented a new line of research that was outside the scope of the monitoring activity. Additional investigations could not be justified because of the absence of funding for such activities.

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Subject: Response to your OIA Request - Offal holes at Ruakura

[redacted]

I am writing in response to your Official Information Act request dated 7th May 2010 for information on the numbers and sizes of the offal holes located at the Ruakura site; the ground where the cows are destroyed and buried "the offal pits".

Q1. How many offal pits are in use?
There are no "offal pits"; there are currently three active offal holes, which are surrounded by a 200mm concrete riser and have a concrete lid to prevent external water entry.

Q2. How many offal pits have been sealed over and how has this been done?
There have been 27 offal holes over 10yrs. They have had excess dirt mounded on top to seal and these are monitored and any subsidence is corrected by mounding more dirt on top.

Q3. What is the depth of the offal pits?
Offal holes are drilled to a depth of approximately 7m.

Q4. What is the diameter of the offal pits?
Offal holes are 900mm in diameter and the concrete risers and lids described above are 1200mm.

Yours sincerely

[redacted]