1. Validation steps that have been carried out and 'passed'

1	In silico analysis	PCR protocol applied but not optimised					
2	In silico analysis	PCR Reaction optimised	Some in vitro testing of related species suggests test is specific	Detection obtained from water samples in laboratory environment or mesocosm			
3	In silico analysis	PCR Reaction optimised	In vitro specificity confirmed – all closely-related co-occurring species tested	Extensive field testing at sites of known presence and absence of target species			
4	In silico analysis	PCR Reaction optimised	In vitro specificity confirmed – all closely-related co-occurring species tested	Extensive field testing at sites of known presence and absence of target species	Limits of detection established		
5	In silico analysis	PCR Reaction optimised	In vitro specificity confirmed – all closely-related co-occurring species tested	Extensive field testing at sites of known presence and absence of target species	Limits of detection established	Detection probability estimates from statistical modelling	Good understanding of ecological, temporal and spatial factors affecting detectability

2. Remaining uncertainties at each level

1	2	3	4	5
DNA may have been introduced by other organisms, including humans. If the target is also a food item then it could have been introduced via waste water	DNA may have been introduced by other organisms, including humans. If the target is also a food item then it could have been introduced via waste water	DNA may have been introduced by other organisms, including humans. If the target is also a food item then it could have been introduced via waste water	DNA may have been introduced by other organisms, including humans. If the target is also a food item then it could have been introduced via waste water	DNA may have been introduced by other organisms, including humans. If the target is also a food item then it could have been introduced via waste water
It is not known how ecological and seasonal factors may influence detectability	It is not known how ecological and seasonal factors may influence detectability	It is not known how ecological and seasonal factors may influence detectability	It is not known how ecological and seasonal factors may influence detectability	Some untested ecological factors may still influence detectability
It is not known how many samples are required for a 95% chance of detection	It is not known how many samples are required for a 95% chance of detection	It is not known how many samples are required for a 95% chance of detection	It is not known how many samples are required for a 95% chance of detection	Sensitivity may vary in different environments
Co-occurring species that have not been tested may co-amplify, leading to false positive results. PCR products should be sequenced to verify target presence	Co-occurring species that have not been tested may co-amplify, leading to false positive results. PCR products should be sequenced to verify target presence	Co-occurring species that have not been tested may co-amplify, leading to false positive results. PCR products should be sequenced to verify target presence	Unrelated species may occasionally co-amplify, although the risk is low given the extensive field testing undertaken. Additional specificity testing may need to be undertaken if using the assay in a different region to that in which it was originally validated	Unrelated species may occasionally co-amplify, although the risk is low given the extensive field testing undertaken. Additional specificity testing may need to be undertaken if using the assay in a different region to that in which it was originally validated
It is not known if this assay will work on environmental samples	It is not known if this assay will work on environmental samples			

3. Valid interpretation of results

	1	2	3	4	5
Negative Result	Impossible to tell if target is present or absent	Impossible to tell if target is present or absent	Impossible to tell if target is present or absent	Target is likely to be absent, assuming sampling has been carried out at an appropriate time of year and with a high level of replication	Target is likely to be absent. Assuming appropriate sampling has been carried out, a probability of species presence (false negative) can be given
Positive Result – Amplification	Impossible to tell if target is present or absent	Impossible to tell if target is present or absent	Impossible to tell if target is present or absent	Target is very likely to be present	Target is very likely to be present
Positive Result – Sequenced	DNA of target is definitely present	DNA of target is definitely present	DNA of target is definitely present	DNA of target is definitely present	DNA of target is definitely present