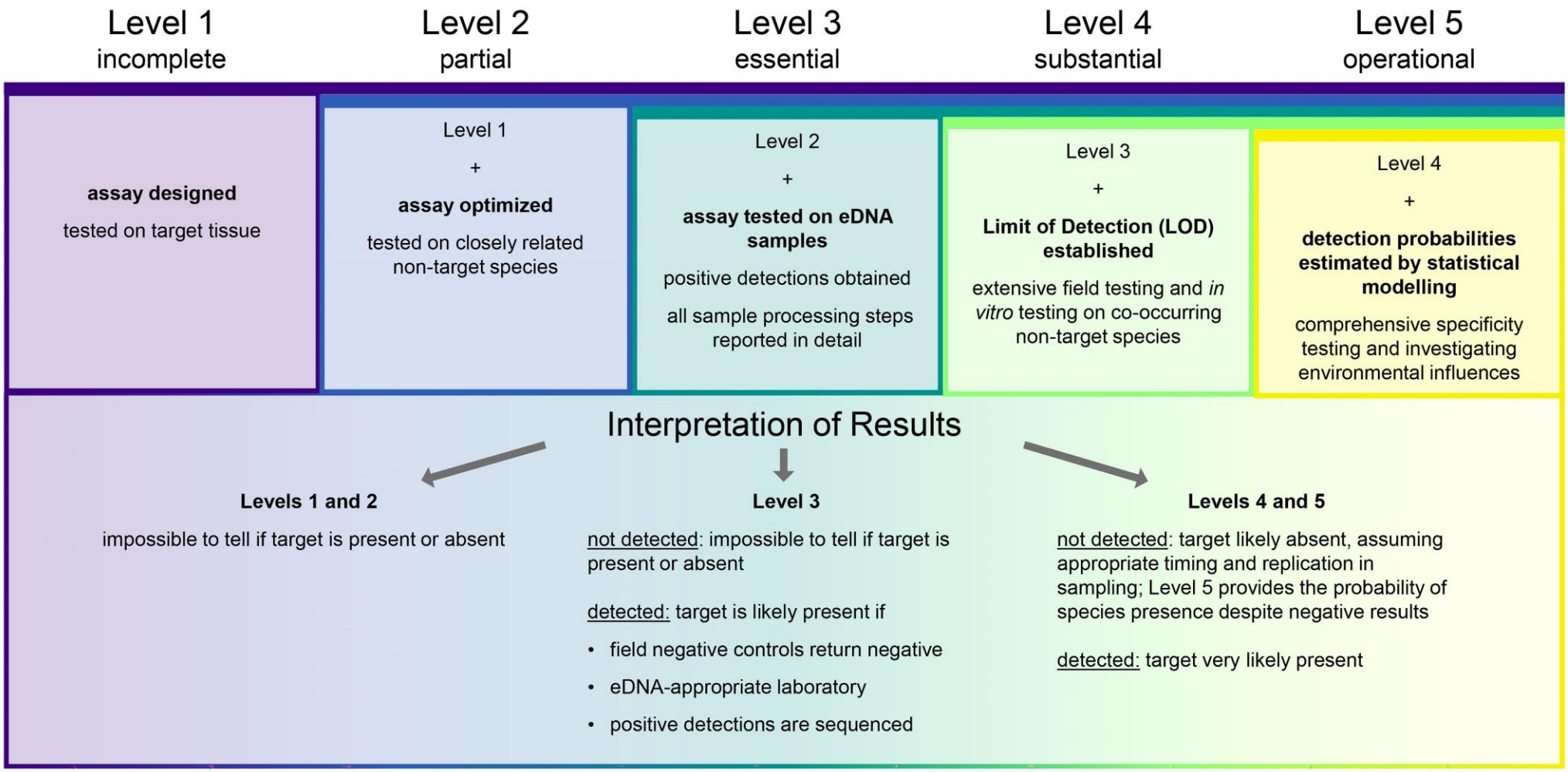
The validation scale



Variable blocks and minimum criteria

Validation level	Variable blocks	Minimum criteria
Level 1	*in silico* analysis	target species
	target tissue testing	target tissue
	target tissue PCR	primer (and probe) sequence
Level 2	comprehensive reporting of PCR conditions	DNA extract volume in PCR
	in vitro testing on closely related non-target species	any in vitro non-target testing
Level 3	extraction method performed on eDNA samples	method of extraction
	concentration of eDNA from environmental sample	filter type or precipitation chemicals
	detection obtained from environmental samples	detection from an environmental sample (artificial or natural habitat)
Level 4	Limit of Detection (LOD)	LOD determined
	extensive field testing of environmental samples	multiple locations or multiple samples
	in vitro testing on co-occurring non-target species	any advanced *in vitro* testing
Level 5	comprehensive specificity testing	non-co-occurring/closely related species checked from *in silico*
	detection probability estimation from statistical modelling	any effort made towards detection probability estimation
	understanding ecological and physical factors influencing eDNA in the environment	any factor influencing eDNA in the environment tested

Remaining uncertainties at each level

Level 1	Level 2	Level 3	Level 4	Level 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.			

Valid interpretation of eDNA-based results

	Level 1	Level 2	Level 3	Level 4	Level 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	Target is likely to be present	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

***Contamination is always a possibility; therefore, enough negative controls need to be generated from field sampling to PCR along the whole processing chain. This is especially important if short target DNA fragments (below 100 base pairs) are amplified, and the sequenced fragment consists mainly of primer sequences and does not allow for discrimination between species.