Working Outline for White Paper on Detection and Monitoring of Invasive Species by Polymerase Chain Reaction (PCR)

From: ISAC Early Detection & Rapid Response Subcommittee
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PURPOSE: At the June 2010 Invasive Species Advisory Committee meeting, the Early Detection, Rapid Response Subcommittee (EDRRSC) committed to develop a working outline of a future white paper on detection and monitoring of aquatic invasive species with PCR techniques.

BACKGROUND: Early detection and monitoring of invasive aquatic species is critical to successful eradication and control efforts, as aquatic species in particular are often difficult to detect once they have spread beyond confined waters. A new detection method was presented by Ficetola et al. (2008), where persistence of an invasive amphibian's DNA in the environment (eDNA) was detected with polymerase chain reaction (PCR) amplification. PCR is both simple and complex. The need to improve the reliability of PCR assays is being voiced by authors who have explored the various attributes of species detection assays, such as those for *Dreissena* spp. (Frischer, Nierwicki-Bauer and Kelly, 2011) and Asian carp (Darling and Mahon 2011). While those investigating the utility and accuracy of PCR are raising concerns, the array of the PCR tools continues to expand for invasive species across the country. The PCR paradigm shift, which includes unprecedented sensitivity potential, time savings, and widespread application, makes a very tempting situation for regulators. In addition to assay validation, agencies responsible for managing AIS speak of a need to verify the performance of individual laboratories. Regulators must have a counter-balanced perspective with validation of an assay and laboratory accreditation so the results of each assay are reflective of the real situation sampled. The stakes are enormous when being charged with protecting our nation's security in terms of maintaining biodiversity; and the country's economy, animal/plant health, environment, and public health. Proper and adequate validation is the essential prerequisite to ensure that the promise of PCR for detection and monitoring of invasive species can be fulfilled. To be useful in decision-making, assay performance must be evaluated before testing the samples.

OUTLINE: The four primary issues to be included in the white paper(s) are: 1) PCR technology, 2) the assay validation/optimization process and, 3) Laboratory accreditation/standardization, and 4) Regulatory use.

1) PCR Technology

Terms and definitions – See Appendix A for complete glossary of terms used

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2) Assay Validation and Optimization

Overview – Trade issues/Environmental preservation/public health/economic
"Designer assays" thru assay validation - Define end use before beginning
Assay parameters defined
Assay specificity
"Satisfactory test" defined/specified
Controls defined/specified extraction suitable, cross contamination
Standards defined/specified
Traceable reagents
Equipment required and specifications
Personnel qualifications specified
Record integrity specified (record change/document control) – include electronic systems
Actions to take w/ false positives or false negatives suspected
Valid Test - Then you can say positive or negative
Screening – w/ high sensitivity
Have to know your false positive rate
Confirmatory – w/ high specificity
Research is different than Diagnostic
Diagnosticians want yes/no answer
Researchers want to go off and retest or adjust.
No test
Repeat without prejudice
Retest
Permitted
Prohibited
Variable interpretation (States w/ different interpretations; Trade requirements; national; etc.)
Contamination, inhibitors, enhancers, etc.
Assay Optimization

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3) Laboratory Accreditation and Standardization

International Accreditation Standards in existence
International Committee on Harmonization (ICH)
ISO/IEC 17025
National Accreditation Standards in USA

There are a large number of laboratory certification programs
chemical, [does this item need to be oriented to inorganic process or manufacturing tests?]
biological, [does this item need to orient towards organic science?]
animal health, [supporting trade agreements covering animal, enviro-, public health, economic health?]
public health [should this orient towards national security, bioterrorism, etc]
forensic science [w/ use in court cases, etc.]

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4) Regulatory Use and License to Use – approval for field use [Comment: Need further information here]
There is a need for useful regulatory authority, framework and oversight for assay use.
Informing and educating regulators to need for validated assays and accredited laboratories
Uniform methods & regulations
Validation framework (i.e. International Committee on Harmonization, ISO/IEC 17025)
Validation must come first
Official review after validation
Use after regulatory approval, license, certification, etc.
Example Groups offering accreditation:
The American Association of Lab Accreditation
Lab Accreditation Bureau
National Cooperation for Laboratory Accreditation
The American Society of Crime Laboratory Directors - Laboratory Accreditation Board
National Animal Health Laboratory Network (NAHLN). Animal Health: Testing for certain types of diseases must be performed at either the National Veterinary Services Laboratories (NVSL) or other APHIS-approved facilities.

The development of a DNA-based AIS detection tool requires both an understanding of technology limitations and field use conditions. Such understanding will guide appropriate assay design and validation. Defining Standard Testing Practices (STP's) will greatly improve the supporting information for regulatory decisions. Such advice is offered recently in Darling and Mahon 2011, and other authors awakening to this new reality.

"Validation is the bridge between research and regulatory decisions!"
(Anything else is jumping across the abyss of unknowns to any possible conclusion!)

Literature Cited

Darling, J.A. and A.R. Mahon. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. *In press*. Environmental Research. January 2011. DOI:10.1016/j.envres.2011.02.001.

Ficetola, G. F., C. Miaud, F. Pompanon, and P. Taberlet. 2008. Species detection using environmental DNA from water samples. Biology Letters 4:423-425.

Frischer, M. E., Nierzwicki-Bauer, S. A. and K.L. Kelly. 2011. Reliability of Early Detection of *Dreissena* spp. Larvae by Cross Polarized Light Microscopy, Image Flow Cytometry, and Polymerase Chain Reaction Assays Results of a Community Double-Blind Round Robin Study (Round Robin Study Phase II). Available at http://www.musselmonitoring.com/Reports/RRII%20Final%20Report%20%282010%29.pdf

APPENDIX A

Glossary of Terms used in Assay Validation

Note: The following definitions are intended for use in the context of Polymerase Chain Reaction assays and perhaps similar bioanalytical methods. Not all definitions will be consistent with terminology from all disciplines utilizing steps of sampling, testing, reporting, etc., as discussed here[1].

Accuracy –the closeness of mean test results obtained by the method to the true value (theoretical or accepted reference) of the analyte. This is sometimes referred to as *Trueness* or *Bias*. Refer to the FDA Guidance on Bioanalytical Method Validation (May, 2001),
- Nearness of a test value to the expected value for a reference standard reagent of known activity or titer.[2]

Anaylte - component of the sample, which if present, will be measured within the capabilities of the assay or analytical method to determine presence or define the degree of presence depending on the assay method validated. The assay result can only speak to the sample contents.

Assay or *Assay Method* – See *Test Method*.

Assay Platform - Technology used to measure analyte presence. (e.g. Fluorescence detection or Radiometric counting).

Assay optimization - The process of developing an assay (prior to validation) wherein the variables affecting the assay are elucidated (e.g., Analyte concentration, incubation time, wash cycles, etc.). This process is ideally carried out using a multi-variate factorial approach where the inter-dependence between multiple variables/parameters can be taken into account.[3]

Assay sensitivity – measures the proportion of actual positives which are correctly identified in the positive group.[4] See *Sensitivity*.

$$\text{sensitivity} = \frac{\text{number of True Positives}}{\text{number of True Positives} + \text{number of False Negatives}}$$

Assay specificity - measures the proportion of negatives which are correctly identified in the negative group.[5] See *Specificity*.

$$\text{specificity} = \frac{\text{number of True Negatives}}{\text{number of True Negatives} + \text{number of False Positives}}$$

Assay validation - is the confirmation via extensive laboratory investigations that the performance characteristics of an assay are suitable and reliable for its intended analytical use. It describes in mathematical and quantifiable terms the performance characteristics of an assay. [6]

-the term *validation* should be used for the final decision as to whether the performance criteria justify the application of the test in a given situation.[i]

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[1] Jean W. Lee, et al, Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement, Pharmaceutical Research, Volume 23, No. 2, February 2006

[2] OIE Terrestrial Manual, Glossary of Terms, http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407

[3] http://assay.nih.gov/assay/index.php/Section18:Glossary – accessed 20110407

[4] http://en.wikipedia.org/wiki/Sensitivity_%28tests%29 - accessed 20110407

[5] ibid.

[6] Jean W. Lee, et al, Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement, Pharmaceutical Research, Volume 23, No. 2, February 2006

- is the process of demonstrating and documenting that the performance characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use.[7]

Basic/exploratory research - is conducted to identify unknowns or potential hazards, elucidate the mode/mechanism of action for known characteristics, or explore novel end points for possible subsequent formal validation.[ii] These studies commonly employ sampling methods or samples not relevant for field use; include few groups and few samples per group, and/or nonvalidated end points; are not traceable to adverse outcomes; and are typically creative, short term, relatively inexpensive, and funded by universities, government grants, and nongovernmental organizations (NGOs). These basic research studies play significant roles but are limited in assessing potential for field use. Compare to *Guideline-compliant studies*.

Bias – see *Accuracy*.

Chain of custody – the defined protocol for sample collection, handling, shipment, and storage that maintains both the integrity of the sample itself and the integrity or quality of the analyte to be measured. The Chain of custody protocol will specify the responsible/qualified persons, possibly an official-list of steps by the authority over sighting the sampling, documentation required, and necessary security to prevent tampering or otherwise altering a sample between collection and testing.

Confirmatory test – Assay method(s) of high diagnostic specificity that are used to confirm results, usually positive results, derived
from other test methods.[8]

Controls - Also see *Standards*.

Dynamic Range - the interval between the upper and lower concentration of the analyte in the sample for which the assay has been demonstrated to have acceptable level of accuracy, precision, linearity, etc.[9]

False Negative - Negative reactivity in an assay of a test sample obtained from an animal exposed to or infected with the organism
in question, may be due to lack of analytical sensitivity, restricted analytical specificity or analyte degradation,
decreases diagnostic sensitivity.[10]

False Positive – an assay result that is not indicative of the target analyte.[11] The sources of false positives include, random or systematic errors in handling, spectrophotometric or fluorescence interference of the assay signal by chemical compounds, reagent instability etc. It is important to note that false positives can be reproducible when they are not related to random errors (as in the case of compound interference).

- Positive reactivity in an assay that is not attributable to exposure to or infection with the organism in question, maybe due to immunological cross-reactivity, cross-contamination of the test sample or non-specific reactions, decreases diagnostic specificity.[12]

Fitness for Use – test methods and related procedures must be appropriate for specific field applications in order for the test results to be of any relevance or value to making decisions.[13]

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[7] Biological Assay Validation - http://www.usp.org/pdf/EN/2010-03-25_1033_PF36%284%29_w_line_numbers.pdf - accessed 20110331

[8] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407

[9] Biological Assay Validation - http://www.usp.org/pdf/EN/2010-03-25_1033_PF36%284%29_w_line_numbers.pdf - accessed 20110331

[10] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407

[11] Biological Assay Validation - http://www.usp.org/pdf/EN/2010-03-25_1033_PF36%284%29_w_line_numbers.pdf - accessed 20110331

[12] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407

Gold-standard(s) - refers to a test or benchmark that is the best available under reasonable conditions.[14] A hypothetical ideal "gold standard" test has a sensitivity of 100% with respect to the presence of the analyte (it does not have any false-negative results) and a specificity of 100% (it does not have any false-positive results). In practice, there are sometimes no true "gold standard" tests. The AMA Style Guide prefers the phrase *Criterion Standard* instead of "gold standard".
Good laboratory practices (GLP) - GLPs require complete, permanent documentation of staff; valid study design; standard operating procedures (SOPs); training, performance, formulation, and statistical analyses; and retention of summary/individual data, so there is confidence in the study design, performance, and results, and anyone can subsequently fully reconstruct the study.[15]
Guideline-compliant studies - evaluate potential hazard and risk of substances and are performed following/exceeding governmental regulatory testing guidelines (TGs) and good laboratory practices (GLPs). Guideline-compliant multi-generational reproductive toxicity studies, with large numbers of animals per group per generation, are very expensive and typically funded by manufacturers, consortia of manufacturers, and/or governments. These studies are necessary for hazard evaluation and/or risk assessment because of their statistical power to detect reproducible effects linked to adverse outcomes; relevant exposure routes, doses, and animal models; and dose–response assessment. Compare to *Basic/exploratory research*.
Limit of Detection – See *Sensitivity (analytical)* -
Negative -
Qualitative cutoff –
Quantitative cutoff -
Physical Data – taking of corresponding physical data (pH, temperature, Ca+ concentration, etc.) at the time of sampling as it may be crucial to evaluating PCR outcomes particularly in aquatic invasive species sampling.
Positive -
Precision - A quantitative measure (usually expressed as standard deviation, coefficient of variation) of the random variation between a series of measurements from multiple sampling of the same homogenous sample under the prescribed conditions of the protocol.[16]
Protocol - Complete detailed protocol. All steps, equipment used, all vendor & catalog # for reagents.[17]
Reagent – a substance used to detect; measure another substance; or convert one substance into another by means of the reaction it causes.
Reagent standards or *Standard Reagents* - (specified assay components for testing the analyte, i.e., primers, extraction buffers, etc.)

> *International Standard Reagents* - Standard reagents by which all other reagents and assays are calibrated; prepared and distributed by an International Reference Laboratory.[18]
> *National Standard Reagents* - Standard reagents calibrated by comparison with International Standard Reagents; prepared and distributed by a National Reference Laboratory.[19]
> *Working Standards (reagents)* - Standard reagents calibrated by comparison with the National Standard Reagent, or, in the absence of a National Standard Reagent, calibrated against a well-

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[13] Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.1.04_VALID.pdf - accessed 20110407
[14] http://en.wikipedia.org/wiki/Gold_standard_%28test%29 - accessed 20110407
[15] http://en.wikipedia.org/wiki/Good_Laboratory_Practice - accessed 20110407
[16] See [d] above.
[17] http://ncgc.nih.gov/guidance/HTS_Assay_Guidance_Criteria.html - accessed 20110407
[18] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407
[19] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407

characterised in-house standard reagent; included in routine diagnostic tests as a control and/or for normalisation of test results.[20]

Reagent standardization – the method verifying reagent standards that are specified in the assay protocol of a validated assay.

Receiver operating characteristic (ROC) - is a graphical plot of the sensitivity, or true positive rate, vs. false positive rate (1 − specificity or 1 − true negative rate), for a binary classifier system.[21]

Repeatability - is the precision of repeated measurements within the same analytical run under the same operating conditions over a short interval of time. It is also termed intra-assay or intra-batch precision.

Reproducibility (Run to Run) - A general term to describe the precision of results generated from multiple runs of a compound (or any homogenous test sample) in an assay.

Robustness - Robustness is a measure of the capacity of the assay to remain unaffected by small, but detectable changes in method parameters and provides an indication of its reliability during normal run conditions.[22] PCR is not as robust as ELISA (Enzyme-linked immunosorbent assay) .

Sample or *Specimen* – Material submitted for testing that contains the analyte.[23]

Sample handling – Necessary, defined, validated methods of collection, preparation, shipping, storage, and processing for the assay to be conducted so as to preserve the integrity of the analyte being measured in the sample or submitted specimen.

Sample stability – Expected duration of samples to maintain analyte integrity that represents the sample's origin. This may also be referred to as storage life/expiration dating.

Sampling technique or *Sampling protocol* – (1) the statistical methodology followed to obtain a representative sample of the originating material; and, (2) the defined protocol for locating the sampling site, sample volume, sample handling and shipment, etc.

Screening test – An assay of high *sensitivity (diagnostic)* suitable for large-scale application[24]

Sensitivity - True positive rate or TRP (also see *Assay Sensitivity*) ; TPR = TP / P = TP / (TP + FN)[25]

Sensitivity (analytical) = "Limit of Detection" - smallest detectable amount of analyte that can be measured with a defined certainty.[26]

Sensitivity (diagnostic) Proportion of known infected, affected, or reference origins that test positive in the assay; known infected, affected, or reference origins that test negative are considered to have false-negative results.[27]

Sensitivity (relative) - Proportion of infected, affected, or reference origins defined as positive by one or a combination of test methods that also test positive in the assay being compared.[28]

Specificity = true negative rate or TNP (also see *Assay Specificity*); TNP = TN / N = TN / (TN + FP) = 1 - FPR[29]

Specificity (analytical) - Degree to which the assay distinguishes between the target analyte and other components in the sample matrix; the higher the analytical specificity, the lower the level of false-positives.[30]

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[20] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407

[21] http://en.wikipedia.org/wiki/Receiver_operating_characteristic - accessed 20110407

[22] http://assay.nih.gov/assay/index.php/Section18:Glossary – accessed 20110407

[23] ibid.

[24] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407

[25] ibid.

[26] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407

[27] ibid.

[28] ibid.

[29] http://en.wikipedia.org/wiki/Receiver_operating_characteristic - accessed 20110407

[30] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407

Specificity (diagnostic) - Proportion of known uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results.[31]

Specificity (relative) - Proportion of reference animals defined as negative by one or a combination of test methods that also test negative in the assay being compared.[32]

Standards – Also see *Controls*.

Standard Operating Procedure (SOP) - the *International Conference on Harmonisation* (ICH) defines SOPs as "detailed, written instructions to achieve uniformity of the performance of a specific function".[33]

Standard Reagent - The Standard Reagent is critical in bioassays because its quality offers a reliable material to which a test preparation can be quantitatively compared in an assay.[34]

International Standard Reagents - Standard reagents by which all other reagents and assays are calibrated; prepared and distributed by an International Reference Laboratory.[35]

National Standard Reagents - Standard reagents calibrated by comparison with International Standard Reagents; prepared and distributed by a National Reference Laboratory.[36]

Working Standards (reagents) - Standard reagents calibrated by comparison with the National Standard Reagent, or, in the absence of a National Standard Reagent, calibrated against a well-characterised in-house standard reagent; included in routine diagnostic tests as a control and/or for normalisation of test results.[37]

Test Method or *Assay* - Specified technical procedure for detection of an analyte.

Trueness - see *Accuracy*.

Glossary of Terms used in Laboratory Accreditation

Equipment calibration -

Equipment specification –

Integrity of assay results - (properly recorded, reported, and responsive or timely)

Laboratory accreditation –

Laboratory qualification – see *Laboratory accreditation*

Interlaboratory experiments - the different kinds of interlaboratory experiments depend on the aim for which they are planned.[38]

Collaborative trial or *Method Performance Study* [iii]– a study when the performance of a single method has to be tested

Proficiency testing or *Laboratory Performance Study* [iv] - the comparison of different laboratories that perform comparable analyses with their own individual methods

Round robin study[v] – See *Proficiency testing* or *Laboratory Performance Study*.

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[31] ibid.

[32] ibid.

[33] http://en.wikipedia.org/wiki/Standard_operating_procedure - accessed 20110407

[34] Design and Development of Biological Assays - http://www.usp.org/pdf/EN/2010-03-25_1032_PF36%284%29_w_line_numbers.pdf – accessed 20110331

[35] http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.04_GLOSSARY.pdf - accessed 20110407

[36] ibid.

[37] ibid.

[38] A. Jurado-López · M. D. Luque de Castro, An atypical interlaboratory assay, Anal Bioanal Chem (2002) 372 :109–114

Reproducibility (Lab to Lab) - Reproducibility across labs expresses the precision between laboratories. It is useful for assessing the "transferability" of an assay and/or the validity of comparing results from samples that are run in two or more laboratories.

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[i] John R. Crowther, Methods in Molecular Biology, The ELISA Guidebook, Vol. 516

[ii] Tyl RW 2009. Basic Exploratory Research versus Guideline-Compliant Studies Used for Hazard Evaluation and Risk Assessment: Bisphenol A as a Case Study. Environ Health Perspect 117:1644-1651. doi:10.1289/ehp.0900893

[iii] ISO 5725-2-1994, Geneva, 1994, International Standard, Accuracy (Trueness and precision) of measurement methods and results - Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method,

[iv] ISO/IEC Guide 43-1: 1997 (e), Proficiency testing by interlaboratory comparisons: Part 1: Development and operation of proficiency testing schemes

[v] Misra RK, Uthe JF, Musial CJ (1992) Analyst 117:1085–1091