Validation Assessment of *In Vitro* Lead Bioaccessibility Assay for Predicting Relative Bioavailability of Lead in Soils and Soil-like Materials at Superfund Sites

1. Introduction

Validation and regulatory acceptance criteria articulated in EPA (2007a), as adapted from ICCVAM (1997), have been applied to an *in vitro* lead bioaccessibility (IVBA) assay described in detail in EPA (2007b). This report summarizes the basis for the Agency's determination that the IVBA method for lead has satisfied the validation (and regulatory acceptance) criteria for application of the method in an appropriate regulatory context (articulated in the cover letter to EPA, 2007b). The lead IVBA method provides a tool for characterizing site-specific RBA of lead in soils that is far less resource-intensive than the *in vivo* bioassay methods such as the immature swine bioassay (Casteel *et al*. 1997, 2006; EPA, 2007b).

2. Validation Assessment of the In Vitro Lead Bioaccessibility Assay

This section summarizes information pertinent to each of the validation criteria established in the Agency soil bioavailability guidance EPA (2007a). Because many of the criteria overlap for this assessment, the method validation and regulatory acceptance criteria were consolidated.

2.1. Scientific and regulatory rationale for the test method, including a clear statement of its proposed use, should be available.

The scientific and regulatory rationale for the lead IVBA method is presented in the following documents:

> EPA. (2007a). Guidance for evaluating the bioavailability of metals in soils for use in human health risk assessment. December 2006 OSWER 9285.7-80. May 2007 (Attachment A)

EPA (2007b). Estimation of Relative Bioavailability of Lead in Soil and Soil-like Materials Using In Vivo and In Vitro methods. OSWER 9285.7-77. May 2007. (Attachment B)

Regulatory and scientific rationale: The guidance document (EPA, 2007a) articulates the regulatory rationale for assessing bioavailability of metals soils in assessing human health risks at hazardous waste sites:

> *Accounting for potential differences in oral bioavailability of metals in different exposure media can be important to site risk assessment (U.S. EPA, 1989). This is true for all chemicals, but is of special importance for ingested metals. This is because metals can exist in a variety of chemical and physical forms, and not all forms of a given metal are absorbed to the same extent. For example, a metal in contaminated soil may be absorbed to a lesser extent than when ingested in drinking water or food. Thus, if the oral RfD or CSF for a metal is based on studies using the metal administered in water or food, risks from ingestion of the metal in soil might be overestimated. Even a relatively small adjustment in oral bioavailability can have significant impacts on estimated risks and cleanup goals* (EPA, 2007a).

The guidance also delineates the role of medium-specific bioavailability values intended for use as national default values (i.e., IEUBK Model for Lead in Children, EPA Adult Lead Methodology), from the importance of site-specific values intended to represent conditions at a specific location.

> *However, even in cases where sufficient data exist to support default medium-specific absorption factors for a chemical, site-specific data collection may also be important. Important factors that can affect the bioavailability of metals in soil can be expected to vary from site to site, or within a given site. These include the physical and chemical forms of the metal, as well as the physical and chemical characteristics of the association between the metal and soil particles. Default values for bioavailability may not accurately reflect these factors (e.g., chemistry, particle size, matrix effects) at any given*

> *site. Therefore, use of default values should not substitute for site-specific assessments of bioavailability, where such assessments are deemed feasible and valuable for improving the characterization of risk at the site (see Decision Framework, below)* (EPA, 2007a).

The technical support document (EPA, 2007b) describes in detail two methods that can be used to assess site-specific relative bioavailability (RBA) of lead in soils: 1) an *in vivo* RBA assay in a juvenile swine model; and 2) an *in vitro* bioaccessibility assay (IVBA). The term RBA refers to the ratio of the bioavailability of lead in the soil to that of water soluble lead (e.g., lead acetate). This report summarizes the results of studies that evaluate the validity of the IVBA assay to reliably predict RBA for a range of soil/lead mineral compositions found at lead mining and smelting sites.

The scientific rationale and intended use of these methods are articulated in the technical support document:

> *When reliable data are available on the absolute or relative bioavailability of lead in soil, dust, or other soil-like waste material at a site, this information can be used to improve the accuracy of exposure and risk calculations at that site. Based on available information in the literature on lead absorption in humans, the U.S. Environmental Protection Agency (U.S. EPA) estimates that relative bioavailability of lead in soil compared to water and food is about 60%. Thus, when the measured RBA in soil or dust at a site is found to be less than 60%, it may be concluded that exposures to and hazards from lead in these media at that site are probably lower than typical default assumptions. Conversely, if the measured RBA is higher than 60%, absorption of and hazards from lead in these media may be higher than usually assumed* (EPA, 2006b).

2.2. Relationship of the test method endpoint(s) to the endpoint of interest must be described

The technical support document (EPA, 2007b) describes the outcomes of studies conducted in immature swine to measure RBA for lead, and corresponding IVBA measurements, on 19 soil

samples collected from 8 different mining and smelting sites in EPA Regions 3, 7, and 8. In addition, 2 prepared materials were analyzed, including a Galena-enriched soil and a NIST paint standard. The sources of the samples are identified in Table 2-3 of EPA (2007b). The mineral composition and mineral phase of the lead in the samples (presented in Table 2-4 of EPA, 2007b), varied considerably and are thought to provide a reasonable representation of lead residues expected at residential soils and slag-impacted soil at lead and smelting sites.

2.3. A detailed protocol for the test method must be available and should include a description of the materials needed, a description of what is measured and how it is measured, acceptable test performance criteria (e.g., positive and negative control responses), a description of how data will be analyzed, a list of the materials for which the test results are applicable, and a description of the known limitations of the test including a description of the classes of materials that the test can and cannot accurately assess.

Standard Operating Protocol (SOP): A detailed description of the IVBA method and the statistical approaches used in the assessment of prediction limits of the assay (see Section 2.2) is provided in EPA (2007b). A stand-alone Standard Operating Protocol (SOP) has also been developed by the Agency (EPA, 2008).

Applicable test materials: Application of the IVBA method SOP is expected to yield predictions of RBA that fall within the prediction interval of the assay (EPA, 2007b; see Section 2.2 of this report). The prediction interval was based on results of assays of samples having a wide range of different soil types and lead phases from a variety of different sites. However, most of these samples tested were from a mining and milling sites, and it is possible that IVBA assay results of some forms of lead that do not occur at this type of site might fall within the established prediction interval. Therefore, whenever a sample containing an unusual and/or untested lead phase is evaluated by the IVBA protocol, this should be identified as a potential source of uncertainty in the resulting prediction of RBA. In the future, as additional samples with a variety of new and different lead forms are tested by both *in vivo* and *in vitro* methods, the range of applicability of the method may be further refined.

Assay limitations: Limitations of regulatory applications of the IVBA assay are identified in the Agency cover letter to the method technical support document (EPA, 2007b). These include the following limitations specific to the IVBA assay:

1. **Application to children and extrapolation to adults**. The IVBA assay was developed to predict lead RBA in children and was calibrated with estimates of RBA made from studies conducted in juvenile swine (EPA, 2007b). The juvenile swine bioassay has been utilized as an experimental methodology for predicting RBA in human children; therefore, the prediction equations for estimating RBA from results of the IVBA assay are assumed to apply to human children. While there is evidence to indicate that absolute bioavailability of soluble lead (e.g., in food or water) varies with age, the Agency is not aware of information on the age-dependence (or independence) of the RBA for lead in soil.

2. **Sample lead concentration limits:** The 19 samples tested in the development of the prediction equation and prediction interval for the IVBA assay described in EPA (2007b) ranged from 1,200-14,000 ppm lead. This validation range should be sufficient for most applications of the methodology. Although there is no basis for predicting that errors would necessarily be introduced into the estimates of RBA if sample concentrations outside this range were used in the IVBA assay, use of such samples without validating comparisons with results of the in vivo juvenile swine assay will introduce additional uncertainty into estimates of RBA. A further constraint on the lead concentration is noted in the attachment; sample concentrations used in the IVBA assay should not exceed 50,000 ppm for relatively soluble forms of lead (i.e., lead acetate, lead oxide, lead carbonate), in order to avoid saturation of the extraction fluid. However, applications of the IVBA assay to such high lead concentrations is unlikely to be relevant for improving risk management decisions; thus, this limitation is not likely to be a serious constraint for use of the methodology. Should additional data become available that would suggest modification of the above limits, the Agency will issue additional guidance. In addition, the minimum soil concentration in the sample is determined by that which is measurable in the assay using the SOP.

3. **Particle size:** All samples tested in the development of the prediction equation and prediction interval for the IVBA assay described in EPA (2007b) were sieved through a 60 mesh screen which excluded particles greater than 250 μm. Particle size can be expected to affect dissolution rates for lead that is embedded in particles and is known to affect absolute bioavailability of lead. Therefore, additional uncertainty will be associated with RBA estimates based on application of the IVBA assay to samples having particle sizes larger than 250 μm. In general, humans are believed to ingest particles that are predominantly smaller than 250 μm in diameter (Kissel *et al*., 1996; Sheppard and Evenden, 1994; Driver *et al*.,1989; Duggan and Inskip, 1985; Que Hee *et al*., 1985; Duggan, 1983), so measures of RBA on samples more coarse than this would usually not be considered relevant to risk assessment. Likewise, RBA estimates based on *in vitro* bioaccessibility assays of samples that have not been processed through a 60 mesh (or finer) sieve are generally not appropriate for quantitative use in site-specific risk assessments.

4. **Soil mineralogy:** The IVBA assay prediction equation for RBA (i.e., Equation 3, see Section 2.2 of this report) is expected to be widely appropriate to a variety of soil types and lead mineral phases. However, most of these samples tested were from a mining and milling sites, and it is possible that IVBA assay results of some forms of lead that do not occur at this type of site might fall with in the established prediction interval. Thus, whenever a sample that contains an unusual and/or untested lead phase is evaluated by the IVBA assay, this should be identified as a potential source of uncertainty.

   Available data are not yet sufficient to establish reliable quantitative estimates of RBA for each of the different mineral phases of lead that are observed to occur in the test materials. However, multivariate regression analysis between point estimate RBA values and mineral phase content of the different test materials allows a tentative rank ordering of the phases into three semi-quantitative tiers (low, medium, or high RBA), as follows:

Low Bioavailability	Medium Bioavailability	High Bioavailability
Fe(M) Sulfate Anglesite Galena Pb(M) Oxide Fe(M) Oxide	Lead Phosphate Lead Oxide	Cerussite Mn(M) Oxide
(M) = metal		

5. **Uncertainty in predicted RBA value:** As noted above, the IVBA assay for lead (U.S. EPA, 2007a) measures IVBA for a test material, and converts this to an estimate of RBA by application of a mathematical formula. The resulting prediction of RBA should be thought of as the best estimate of the central tendency estimate of RBA associated with that IVBA, but the actual RBA (if measured *in vivo*) might be either higher or lower than the prediction, due either to authentic inter-sample variability and/or to measurement error in RBA or IVBA. In general, the best estimate of RBA is the most appropriate value for use in the IEUBK model, but risk assessors and risk managers should use their professional judgment to decide if calculations using other values from within the RBA prediction interval should also be evaluated as part of an uncertainty analysis.

2.4. The extent of within-test variability and the reproducibility of the test within and among laboratories must have been demonstrated. The degree to which sample variability affects this test reproducibility should be addressed.

Within test variability: Precision of the IVBA protocol was assessed with 75 and 83 replicate analyses on each of two standard reference materials (NIST SRM 2710 and 2711, respectively) conducted within one laboratory (University of Colorado at Boulder) over several years. The mean coefficient of variation for both standards was 7% and mean IVBA values (± SD) were 75% ± 5% for SRM 2710 and 84% ± 6% for SRM 2711 (Drexler and Brattin, 2007; EPA, 2007b).

Inter-laboratory reproducibility: An inter-laboratory comparison of performance of the IVBA was conducted with four participating laboratories: ACZ Laboratories Inc.; University of Colorado at Boulder; U.S. Bureau of Reclamation Environmental Research Chemistry Laboratory; and National Exposure Research Laboratory (Drexler and Brattin, 2007; EPA, 2007b). Each participating laboratory applied the IVBA method to analyses (in triplicate) of each of the19 test samples used in the assessment of the method prediction equation (i.e., Equation 3, Section 2.2. of this report). Average within-laboratory variability (coefficient of variation, CV) ranged from 1.4 to 6.3% (Drexler and Brattin, 2007). The inter-laboratory coefficient of variation (i.e., CV for estimates from all laboratories, for each sample) ranged from 1.5% to 6.9% (mean: 3.4%) for 17 of the 19 samples (Drexler and Brattin, 2007). Two samples (*California Gulch AV Slag*, *Galena-enriched Soil*) had coefficients of variation of 18.6% and 29.7%. Mean coefficient of variation for all 19 samples was 5.6%.

Effects of sample variability: EPA (2007b) reported a prediction interval for the IVBA assay that was derived based on analysis of samples having a wide range of different soil types and lead phases from a variety of different sites, that are expected to be typical of application of the assay to mining and smelter sites (see Figure 1 and Section 2.2 of this report). The within-laboratory (University of Colorado at Boulder) coefficient of variation ranged from 0.2% to 26.7% (mean: 6.1%) for the 19 samples (based on data presented in Table 3-1 of EPA, 2007b). The high end of the range was impacted by two samples (*California Gulch AV Slag*, CV=17%; *Galena-enriched Soil*, CV=27%). Excluding the latter two samples, the coefficient of variation for the remaining 17 samples ranged from 0.2% to 11.4 % (mean: 4.2%).

2.5. The test method performance must have been demonstrated using reference materials or test materials representative of the types of substances to which the test method will be applied, and should include both known positive and known negative agents.

Performance with reference materials: Precision of the IVBA protocol was assessed with 75 and 83 replicate analyses on each of two standard reference materials (NIST SRM 2710 and 2711, respectively) conducted within one laboratory (University of Colorado at Boulder) over several years (Drexler and Brattin, 2007; EPA, 2007b; see Section 2.4 of this report).

Performance with representative materials: EPA (2007b) reports the prediction interval for the IVBA assay that was derived based on analysis of samples having a wide range of different soil types and lead phases from a variety of different sites, that are expected to be typical of application of the assay to mining and smelter sites (see Section 2.2 of this report).

2.6. Sufficient data should be provided to permit a comparison of the performance of a proposed substitute test with that of the test it is designed to replace.

The IVBA assay is intended to be used as a more cost-effective surrogate to the immature swine bioassay described in EPA (2007b). The 95% prediction interval for IVBA assay predictions of *in vivo* swine bioassay estimates of RBA is reported in EPA (2007b). The prediction interval was established from analyses of 19 samples from 12 different sites, having a wide range of different soil types and lead phases, that expected to be typical of application of the assay to mining and smelter sites (see Section 2.2).

The relationship between the test method endpoint (i.e., IVBA) and the biological effect of interest (i.e., RBA) is described in the form of a mathematical model. Several different mathematical models were tested including linear, power, and exponential. The results are summarized below (methods are detailed in Appendix D of EPA, 2007b):

Model	**a**	**b**	**c**	**R^2**	**AIC**
Linear: $RBA = a + b \cdot IVBA$	-0.028	0.878		0.924	-30.46
Power: $RBA = a + b \cdot IVBA^c$	-0.003	0.978	1.293	0.931	-29.92
2-Parameter Exponential: $RBA = a + b \cdot \exp(IVBA)$	-0.634	0.619		0.936	-33.02
3-Parameter Exponential: $RBA = a + b \cdot \exp(c \cdot IVBA)$	-0.476	0.464	1.225	0.936	-31.11
AIC, Akaike's Information Criterion; R^2, least square coefficient of determination From Appendix D (page D-14) of EPA (2007b).					

All of the models fit the data reasonably well, with the two exponential models fitting slightly better than the linear model. However, the difference in quality of fit between linear and

exponential models was not meaningful in terms of the intended application of the model to the prediction of RBA from results of the IVBA assay. Therefore, the linear model is currently considered to be the preferred model. As more data become available in the future, the relationship between IVBA and RBA can be reassessed and the best-fit model form reconsidered and revised accordingly.

Linear fitting of the data was also performed taking the error in both RBA and IVBA into account; there was nearly no difference in fit. Based on this outcome, the less complex approach (and more transparent) approach, weighted linear regression, was selected to represent the quantitative relationship between RBA and IVBA. This decision may be revisited as more data become available. The currently preferred model is (based on weighted linear regression) is as follows (Equation 3):

$$RBA = 0.878 \cdot IVBA - 0.028 \quad \text{Eq. (3)}$$

The best fit linear model for the data and corresponding 95% prediction interval are shown in Figure 1. Use of Equation 3 to calculate RBA from a given IVBA measurement will yield the "*typical*" RBA value (i.e. central estimate) expected for a test material with that IVBA, and the true RBA may be somewhat different (either higher or lower).

2.7. Data supporting the validity of a test method should be obtained and reported in accordance with Good Laboratory Practices (GLPs).

Data supporting validity of the IVBA assay are reported in detail in EPA (2007b).

2.8. Data supporting the assessment of the validity of the test method must be available for review.

Data supporting the assessment of the validity of the IVBA assay detailed in EPA (2007b) are available online (http://www.epa.gov/superfund/health/contaminants/bioavailability/guidance.htm).

2.9. The methodology and results should have been subjected to independent scientific review.

EPA (2007b), which describes in the IVBA methodology, has undergone extensive review by EPA scientists, was the subject of an EPA-sponsored workshop in April, 2003, and an independent peer review. The IVBA methodology was reported in a peer-reviewed publication (Drexler and Brattin, 2007).

2.10. The method should be time and cost effective.

Based on studies conducted in the validation of the IVBA (EPA 2007b), costs of assessment of a soil sample using the IVBA assay are expected to range from $1/10^{th}$ to $1/20^{th}$ of the costs of the immature swine bioassay. Time requirements for the IVBA assay are expected to range from $1/20^{th}$ to $1/50^{th}$ of that required to conduct the *in vivo* bioassay (i.e., days compared to weeks).

2.11. The method should be one that can be harmonized with similar testing requirements of other agencies and international groups.

Other international agencies (e.g., Canada, United Kingdom, European Union) are pursuing the development of methods for *in vitro* assessment of RBA of lead and of other metals and inorganic contaminants in soil. The IVBA assay described in the technical support document (EPA, 2007a) is directly applicable to these international programs.

2.12. The method should be suitable for international acceptance.

The IVBA assay is suitable for international acceptance.

2.13. The method must provide adequate consideration for the reduction, refinement, and replacement of animal use.

The IVBA assay is intended to replace the use of the immature swine bioassay and, therefore, widespread adoption of the method will decrease use of animals for assessing RBA of lead in soil.

3. Summary

The IVBA assay for lead has been evaluated against validation criteria established in EPA (2007a) for validation of test methods to be used in a regulatory context. All validation criteria established in EPA (2007a) have been satisfied. Scientific and regulatory rationales for the assay have been articulated. Standard Operating Protocols have been established and tested for intra-laboratory precision and inter-laboratory reproducibility. The quantitative relationship between the IVBA assay output and the test method it is intended to replace (i.e., immature swine bioassay) have been established. The description in the method SOP is expected to yield predictions of RBA that fall within acceptable prediction limits for applications in lead site risk assessment. The prediction interval is based on assays of samples having a wide range of different soil types and lead phases from a variety of different sites and, as a result, the method is expected to be widely applicable to soil typically encountered at lead waste sites. Limitations in the regulatory application of the method have been identified. Based on this assessment, EPA considers the IVBA method to be valid for predicting RBA of lead in soils in support of site-specific risk assessments. The Agency supports and encourages use of this methodology when implemented in context with the decision framework described in its soil bioavailability guidance (EPA, 2007a).

4. References.

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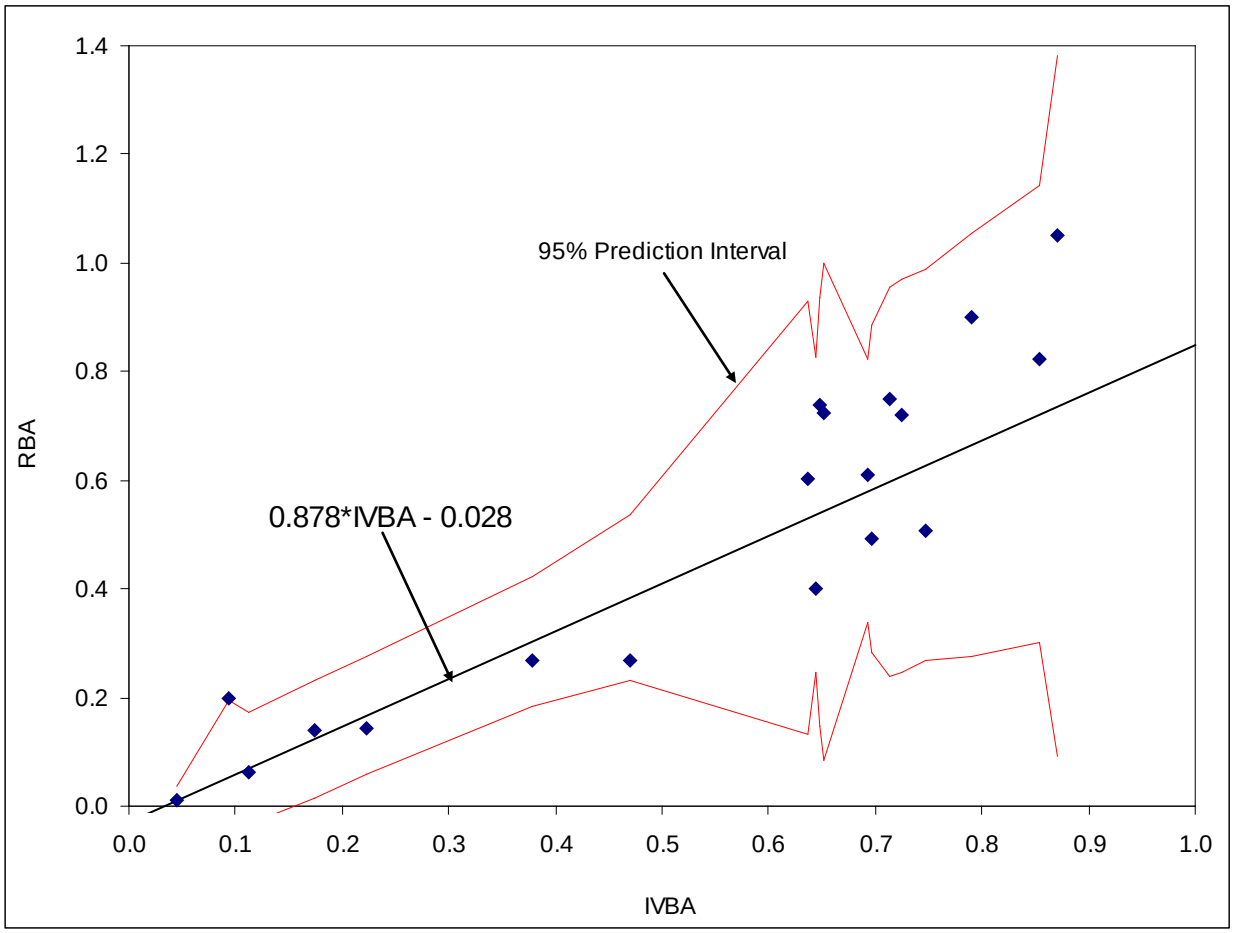

Figure 1. Prediction interval for *in vivo* RBA based on measured IVBA (from Figure D-7 of EPA, 2007b).