Laboratory Procedure Manual

Analyte: **Indoor Allergens**

Matrix: **Dust**

Method: **Multiplex Array for Indoor Allergens (MARIA)**

Method No:

Revised:

as performed by: Indoor Biotechnologies

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Important Information for Users

Indoor Biotechnologies, Inc. periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

File Name	Variable Name	SAS Label
ALDUST_D	DSXAA1	Alt a 1 Result (ng/mL)
	DSXBG2	Bla g 2 Result (ng/mL)
	DSXCF1	Can f 1 Result (ng/mL)
	DSXDF1	Der f 1 Result (ng/mL)
	DSXDP1	Der p 1 Result (ng/mL)
	DSXFD1	Fel d 1 Result (ng/mL)
	DSXMM1	Mus m 1 Result (ng/mL)
	DSXRN1	Rat n 1 Result (ng/mL)

1.   **SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE**

The Multiplex Array for Indoor Allergens (MARIA) is an allergen detection assay developed by Indoor Biotechnologies. The assay is based on Luminex xMap® technology that utilizes beads with unique ratios of internal fluorescent dyes coupled to allergen-specific antibodies. The beads are mixed with a sample and bind the allergen of interest. A biotinylated detection antibody is added, followed by a streptavidin-conjugated fluorophore. The beads are read by an instrument equipped with lasers to identify each bead set and quantify the fluorescent intensity resulting from the bound allergen. The fluorescence intensity for each allergen is compared to a standard curve to determine the allergen concentration in the sample.

2.   **SAFETY PRECAUTIONS**

MARIA assay materials and reagents present no health or safety risks. A laboratory coat and gloves are worn when performing the assay in accordance with general good laboratory practice.

3.   **COMPUTERIZATION; DATA SYSTEM MANAGEMENT**

Indoor Biotechnologies uses Microsoft Access to log and track sample identification information. Raw data from MARIA analysis is compiled using Bio-Plex Manager 4.1 software. Summary data are exported into Microsoft Excel and a custom Excel macro is used to format allergen concentration results.

Analysis results are saved on a password protected, restricted access company server with daily back-up and weekly offsite archival.

4.   **SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

Samples are handled and stored in a manner to prevent or minimize degradation. Samples are received into a designated area where they are inspected by an analyst. The interior temperature of the shipping package is measured and recorded on the Chain of Custody form (COC) upon arrival. Any sample requiring special attention (i.e. damaged container, no sample identity, errant temperature) is recorded on the COC and immediately reported to the Senior Analyst or Senior Scientist who will notify the client, if required.

Samples are assigned a unique batch ID and accession number that is recorded on the sample container and logged into the Microsoft Access database.

Samples will not be processed for analysis if they contain less than 50µL. The client is contacted and made aware of any sample failing to meet this requirement.

All samples are stored at -20°C.

5.   **PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

6. **PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION**

   a. MARIA buffer is prepared from ACS grade or higher reagents purchased from authorized vendors. Reagent information, including the manufacturer, lot number, open date, and expiration date is recorded in a log.
   b. The allergen standard used in MARIA is prepared at Indoor Biotechnologies using purified natural or recombinant allergens. A set of 12 standard dilutions are prepared for every assay.
   c. High and low quality control samples are prepared from purified natural or recombinant allergens. Quality control sample concentrations fall within the usable part of the standard curve for each allergen and are subject to detailed evaluation prior to use in the assay.
   d. The Bio-Plex instrument is subjected to a start-up (instrument self-check), warm-up (lasers brought to proper operating temperature) and calibration procedure each day prior to analysis.

7. **CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES**

   Bio-Rad calibration and validation kits are used for routine verification of Bio-Plex instrument performance. Bio-Plex instruments are calibrated each day prior to use and when prompted by the instrument for recalibration due to the temperature of the reader changing by more than $2^{o}C$. Detailed instrument validation is performed on a quarterly basis by Indoor Biotechnologies analysts and the instruments are serviced annually by a Bio-Rad service engineer. Records of instrument validation and service are maintained in the calibration logs located next to the instruments.

8. **PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS**

   Assay Preparation

   Fill a pipette basin with assay buffer and use a multichannel pipette to pre-wet each well of the 96 well filter plate with 100µL of assay buffer.

   Preparation of Standards:
   Label twelve 1.5mL polypropylene microcentrifuge tubes 1 through 12 to be used for allergen standard dilutions. Pipette 150µL of assay buffer into tubes 2 through 12. Dilute the standard 1/20 in 300µL of assay buffer in tube 1. For example, the 9-plex assay requires 270µL of assay buffer in tube 1 plus 30µL of NHANES Universal Allergen Standard (ST-NHANES). Mix well by vortexing and transfer 150µL from tube 1 to tube 2. Continue to make 12 total serial doubling dilutions, vortexing the tube and changing the pipette tip after every transfer.

   Preparation of Sample Dilutions (dust extracts):
   Vortex samples vigorously for 30 seconds and then centrifuge at 14,000 rpm (16,000 x g) for two minutes. For each sample (plus one for duplicate), pipet 90µL of assay buffer into four wells across a row of a 96-well microtiter plate. Add 10µL of each sample to the first well of each row. Mix well to achieve a 1/10 dilution and transfer 10µL to the next corresponding well to make a 1/100 dilution. Mix and repeat the 1/10 dilution 2x to obtain 1/1,000 and 1/10,000 dilutions.
   *The 1/1,000 dilution is typically not used for analysis, but only for dilution purposes.

Preparation of Bead Solution:
  Prepare the microsphere solution by vigorously vortexing the NHANES bead stock for one minute. Use a 5mL disposable pipette to add 5.5mL of assay buffer to a 15mL centrifuge tube. Add 135µL of the bead stock. Vigorously vortex the bead mix for 30 seconds.

Remove buffer from the 96 well filter plate by vacuum filtration. Tap the plate on paper towels to remove excess buffer from the plate bottom. Repeat vacuum filtration. Tap plate again on paper towels.

MARIA Assay

Pour the bead solution into a pipette basin and use a multichannel pipette to add 50µL microsphere bead solution to each well.
  Add 50µL of diluted standard to duplicate standard wells.
  Add 50µL of sample dilutions to appropriate wells, duplicating one of the samples for QC analysis.
  Add 50µL of assay buffer to the blank wells.
  Add 50ul of undiluted quality control samples (QC-H and QC-L) to quality control wells.
  Set a multichannel pipette to 50µL and mix all wells vigorously (5 rapid repetitions).
  Incubate for one hour at room temperature in the dark.
Prepare biotinylated antibody solution by first adding 12mL of assay buffer to a pipette basin using a 10mL disposable pipette. Add 182µL of premixed biotinylated antibodies (BI-MRA-P9). Use the 10mL pipette to thoroughly mix the antibody solution.
Remove samples and standards by vacuum filtration. Wash wells 2x by adding 100µL of assay buffer and removing by vacuum filtration.
Add 100µL of the diluted biotinylated detection antibodies to each well. Mix vigorously by pipetting as before and incubate for one hour at room temperature in the dark.
Prepare a 1/240 dilution of the Streptavidin-Phycoerythrin (Invitrogen, 1mg/mL, # S866) by pipetting 12mL of assay buffer into a pipette basin followed by 50µL of Streptavidin-Phycoerythrin. Mix well using the 10mL pipette.
Remove the biotinylated antibody solution by vacuum filtration. Wash wells 2x with 100µL of assay buffer.
Add 100µL of the diluted Streptavidin-Phycoerythrin to each well. Mix vigorously by pipetting and incubate for 30 minutes at room temperature in the dark.
Remove Streptavidin-Phycoerythrin solution by vacuum filtration. Wash wells 2x with 100µL of assay buffer.
Add 100µL of assay buffer and resuspend the beads by pipetting repeatedly, taking care not to create a foam.
Wipe the bottom of the filter plate with a paper towel and read the plate on the Bio-Plex Instrument.

Data Analysis and Handling

Export the Bio-Plex Manager data file into Excel using the program icon on the menu bar, and save to the secure server: data on sbs2k3 > BioPlex Analysis Service > NHANES.
Open the Excel file using the data evaluation macro, Run Analysis 3.0, located at data on sbs2k3 > BioPlex Analysis Service. Select 'enable macros', if prompted.
Use the 'browse for files' option to select the file to be analyzed. Enter the number of allergens tested, the number of dilutions, and the dilution factors (e.g. 10 for 1/10, 100 for 1/100, etc.).
Click the Run tab and the results will open in a new Excel spreadsheet.
The summary sheet created by the macro shows all results generated in the MARIA analysis for each allergen measured for all sample dilutions. It also shows information about whether or not each respective value is based on the usable part of the standard curve. Select the final

results for each sample and allergen.
Enter results for each sample and allergen into the Access Database according to the assigned sample accession number.
Quality Control

   Enter results for the two Quality Control samples (QC-High and QC-Low) into the QC Sample Analysis folder: data on sbs2k3 > BioPlex Analysis Service > NHANES > QC Sample Data. If the concentration of any one allergen in the QC-High or QC-Low sample is +/- three standard deviations or the second of two consecutive +/- 2 standard deviations from the established mean value, the samples must be run again.

   Enter results for the duplicate sample into the MARIA Sample Duplicate Data folder on 'data on sbs2k3 > BioPlex Analysis Service > NHANES > Sample Duplicate Data'. If duplicate sample results do not have a CV of 30% or less, samples must be run again.
Reports are prepared by the Senior Analyst and reviewed by the Senior Scientist who will enter them into the data sheets provided and send them to NCHS via email.

9. REPORTABLE RANGE OF TEST RESULTS

Because multiple dilutions of each sample are analyzed, there is no upper limit to the reportable range of allergen concentrations measured by MARIA. The lower reporting limit is determined by multiplying the limit of detection for each allergen by the lowest sample dilution factor. The reporting limit, although generally consistent, may vary slightly between assays due to the usable part of the standard curve, which is determined for each run of the assay. The reportable limits represent the lowest detectable values in the analysis (i.e., values below the reporting limits are considered below detection).

The following reporting limits are based on a 1/10 sample dilution.

Allergen	Limit of Detection (ng/mL)	Reporting Limit (ng/mL)
Der p 1	0.06	0.6
Der f 1	0.06	0.6
Fel d 1	0.02	0.2
Can f 1	0.06	0.6
Mus m 1	0.01	0.1
Rat n 1	0.02	0.2
Bla g 2	0.98	9.8
Alt a 1	0.02	0.2

10. QUALITY CONTROL (QC) PROCEDURES

Quality control procedures for each plate analyzed include the following:
a. A 12-point standard curve runs in duplicate. Duplicate points in the usable range of the curve will have a CV of less than 15%.
b. High and low   quality control samples in the analytical range of the assay.
c. Three blank wells consisting of assay buffer in lieu of sample.
d. Analysis of one random sample in duplicate on each plate. Criteria for acceptance of the duplicate will be a CV of less than 30%.
e. Analysis of samples at multiple dilutions on each plate.
f. A sigmoidal 5 parameter logistic standard curve was used for the MARIA assays

Indoor Biotechnologies will follow Westward rules for QC data: 1-2s-warning; 1-3s-reject; 2-2s-reject. Non-compliant results are subject to corrective action and repeat analysis of the respective sample units.

11. **REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

If instrument calibration fails, a second attempt to calibrate will be made. If the second effort fails, a service call to BioRad will be placed (the assay can be run on the backup instrument).

If QC results fail to meet acceptance criteria, the plate is rejected and samples are rerun. A root cause analysis will be performed and no assay results will be released until the issue is resolved.

12. **LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

Particulates in the sample or assay buffer may be interpreted by the instrument as "bead aggregates" and cause an error. Precautions are taken to limit this interference and include sterile filtration of the assay buffer (assay buffer assigned a shelf life of two weeks), and sample centrifugation prior to analysis to sediment any particulates.

13. **REFERENCE RANGES (NORMAL VALUES)**

Not applicable to this procedure. Allergen levels in house dust can vary widely.

14. **CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable to this procedure.

15. **SPECIMEN STORAGE AND HANDLING DURING TESTING**

Samples are stored at -20°C. Prior to analysis, samples are removed from the -20°C freezer and allowed to thaw. Once an aliquot has been removed for testing, samples are returned to the
-20°C freezer.

16. **ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no alternative methods for performing test if the system fails. The samples are stored until the method is functioning.

17. **TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable.

18. **TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

All samples are labeled with an identification number that is entered into an Access database upon receipt. Following analysis, samples are stored at -20° C in labeled boxes. Data will be transmitted to NCHS in electronic data files generated by Excel software.
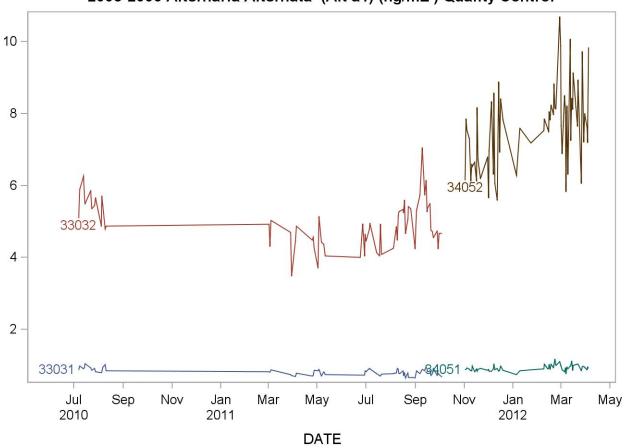
19.   **Summary Statistics and QC Graphs**

See following pages.

Summary Statistics for Alternaria Alternata (Alt a 1) (ng/mL)

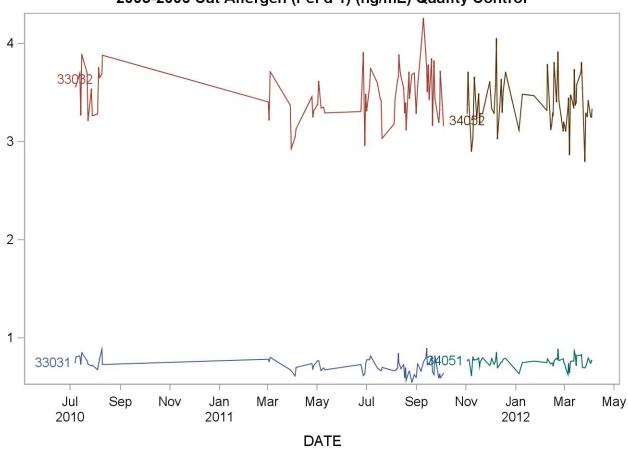| Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
33032	162	07JUL10	04OCT11	4.9707	0.8111	16.3
33031	162	07JUL10	04OCT11	0.8144	0.1063	13.1
34052	164	02NOV11	04APR12	7.5564	1.4346	19.0
34051	164	02NOV11	04APR12	0.9268	0.1111	12.0

2005-2006 Alternaria Alternata (Alt a1) (ng/mL) Quality Control

Summary Statistics for Cat Allergen (Fel d 1) (ng/mL)

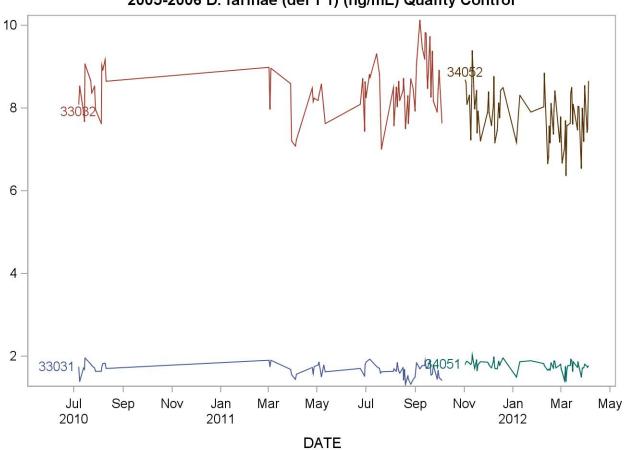| Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
33032	162	07JUL10	04OCT11	3.5006	0.3300	9.4
33031	162	07JUL10	04OCT11	0.7157	0.0828	11.6
34052	164	02NOV11	04APR12	3.3798	0.3348	9.9
34051	164	02NOV11	04APR12	0.7565	0.0720	9.5

2005-2006 Cat Allergen (Fel d 1) (ng/mL) Quality Control

Summary Statistics for D. farinae (Der f 1) (ng/mL)

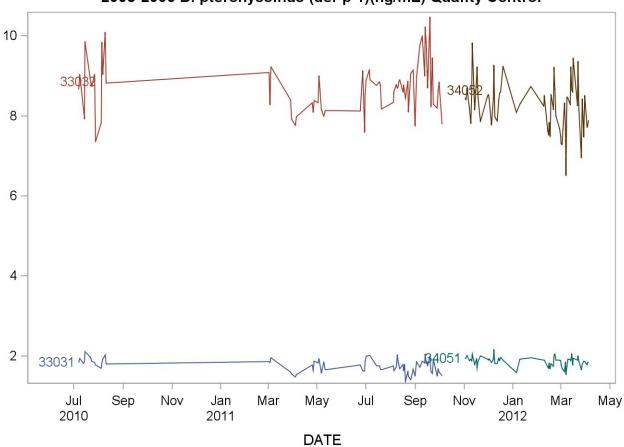| Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
33032	162	07JUL10	04OCT11	8.4260	0.7927	9.4
33031	162	07JUL10	04OCT11	1.6890	0.1849	10.9
34052	164	02NOV11	04APR12	7.7812	0.7894	10.1
34051	164	02NOV11	04APR12	1.7771	0.1573	8.9

2005-2006 D. farinae (der f 1) (ng/mL) Quality Control

Summary Statistics for D. pteronyssinus (Der p 1)(ng/mL)

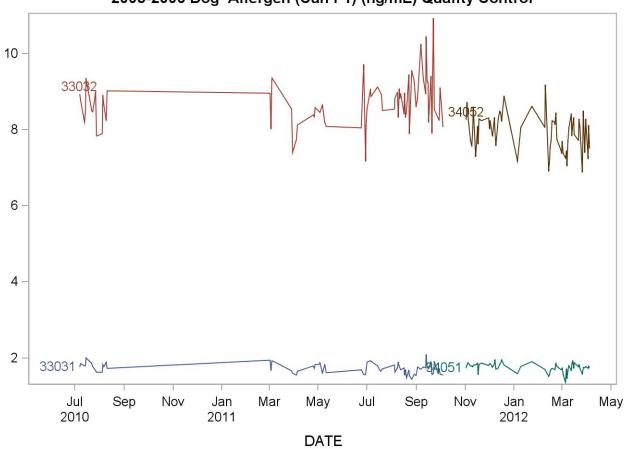| Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
33032	162	07JUL10	04OCT11	8.6972	0.7438	8.6
33031	162	07JUL10	04OCT11	1.7677	0.1875	10.6
34052	164	02NOV11	04APR12	8.2049	0.7970	9.7
34051	164	02NOV11	04APR12	1.8627	0.1676	9.0

2005-2006 D. pteronyssinus (der p 1)(ng/mL) Quality Control

Summary Statistics for Dog Allergen (Can f 1) (ng/mL)

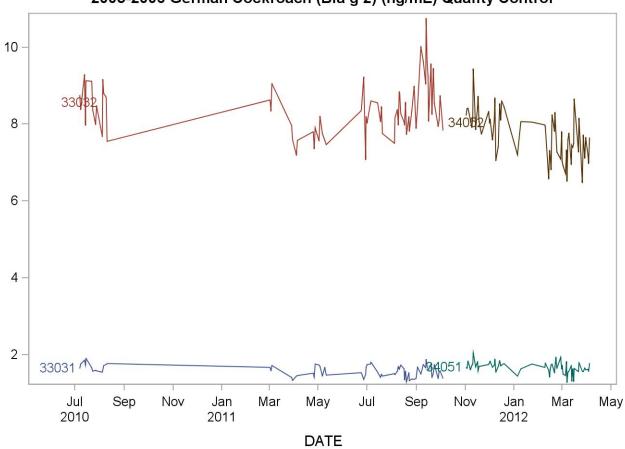| Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
33032	162	07JUL10	04OCT11	8.6637	0.7279	8.4
33031	162	07JUL10	04OCT11	1.7262	0.1529	8.9
34052	164	02NOV11	04APR12	7.9420	0.7062	8.9
34051	164	02NOV11	04APR12	1.7494	0.1596	9.1

2005-2006 Dog Allergen (Can f 1) (ng/mL) Quality Control

Summary Statistics for German Cockroach (Bla g 2) (ng/mL)

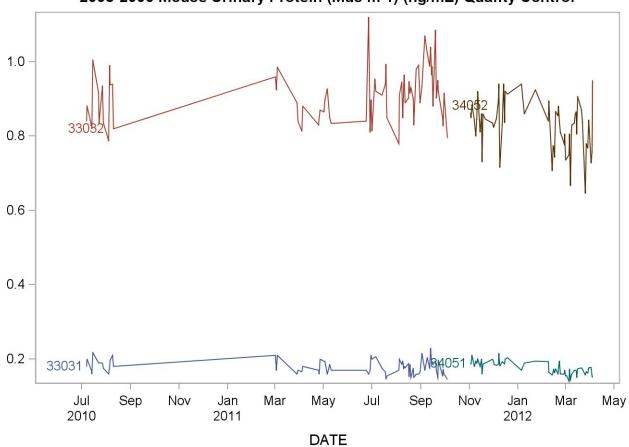| Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
33032	162	07JUL10	04OCT11	8.3584	0.7791	9.3
33031	162	07JUL10	04OCT11	1.5886	0.1723	10.8
34052	164	02NOV11	04APR12	7.6992	0.8262	10.7
34051	164	02NOV11	04APR12	1.6703	0.2205	13.2

2005-2006 German Cockroach (Bla g 2) (ng/mL) Quality Control

Summary Statistics for Mouse Urinary Protein (Mus m 1) (ng/mL)

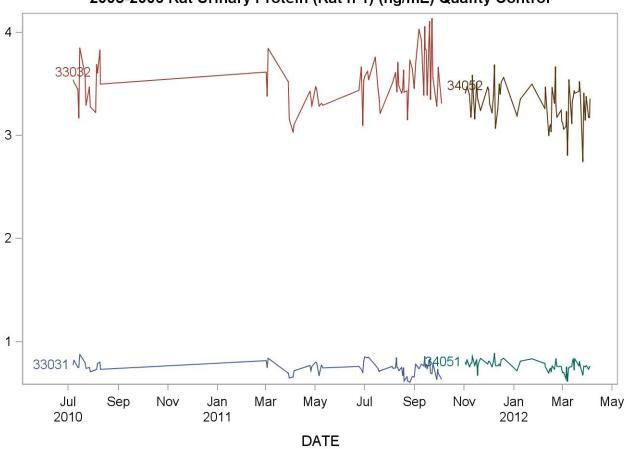| Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
33032	162	07JUL10	04OCT11	0.9085	0.0910	10.0
33031	162	07JUL10	04OCT11	0.1803	0.0225	12.5
34052	164	02NOV11	04APR12	0.8201	0.0891	10.9
34051	164	02NOV11	04APR12	0.1763	0.0214	12.1

2005-2006 Mouse Urinary Protein (Mus m 1) (ng/mL) Quality Control

Summary Statistics for Rat Urinary Protein (Rat n 1) (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
33032	162	07JUL10	04OCT11	3.5006	0.2796	8.0
33031	162	07JUL10	04OCT11	0.7456	0.0719	9.6
34052	164	02NOV11	04APR12	3.2912	0.2499	7.6
34051	164	02NOV11	04APR12	0.7665	0.0647	8.4

2005-2006 Rat Urinary Protein (Rat n 1) (ng/mL) Quality Control

REFERENCES

Earle et al. High-throughput fluorescent multiplex array for indoor allergen exposure assessment. JACI 2007; 119: 428-433