ARBOR ASSAYS™
Interactive Assay Solutions™

DetectX®

Palladium API Screening
Fluorescent Detection Kit

1 Plate Kit Catalog Number K007-F1

Sample Types Validated:

Screening Pd Removal Methods for APIs

Patent Pending

Please read this insert completely prior to using the product.
For research use only. Not for use in diagnostic procedures.

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BACKGROUND

In recent years, many new synthetic transformations have been developed that use palladium (Pd) compounds for the catalysis of carbon-carbon and carbon-heteroatom coupling reactions such as the Buchwald-Hartwig, Heck, Kumada, Negishi, Nozaki-Hiyama, Sonogashira, Stille, Suzuki-Miyaura, and Tsuji-Trost transformations[1,2]. These reactions have found increased popularity for pharmaceutical processes as they utilize a wide-range of functional groups and can therefore be used to build complicated molecules. However, palladium-catalyzed reactions present a problem in that the palladium can often be retained in the isolated product. The LD50 values are very dependent on the physical form of the Pd compounds or catalysts used. For rats, mice, or rabbits, water soluble $PdCl_2$ administered intravenously gave a LD50 of 3 mg/kg body weight while relatively insoluble PdO given orally gave an LD50 >4900 mg/kg body weight[3]. Current European Agency for the Evaluation of Medicinal Products regulations limit all platinum group (Pt, Pd, Ir, Rh, Ru, Os) metal contamination (as a group) to less than 5 ppm[4].

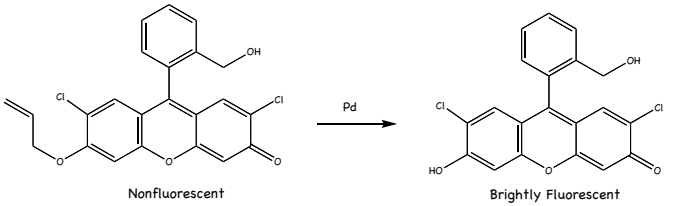
A variety of methods are available for removing Pd from active pharmaceutical ingredients (APIs). These include adsorption with trimercaptotriazine N-acetylcysteine as solids that are removed by filtration[5], on polymeric supports[6] such as polystyrene or as macroporous resins such as the Isolute resins (Biotage)[7]. Other adsorptive methods include activated carbon[8], ion exchange type resins, fiberous materials such as Smopex™ (Johnson Matthey)[9], and silica bound active resins from SiliCycle[10]. Typical chemical purification methods such as crystallization can also be used to lower the concentration of Pd in the final product, especially when combined with additives such as the sulfur containing ligands, N-acetylcysteine[11] and thiourea, and with phosphines to keep the Pd in the mother liquor.

The standard methods of quantifying palladium in APIs are atomic absorption analysis, x-ray fluorescence, and plasma emission spectroscopy such as inductively-coupled plasma mass spectroscopy (ICP-MS)[11]. ICP-MS is typically used both during synthesis and for final QC on drug molecules. These methods require expensive instruments with a highly trained scientist to operate. Cross-contamination of the instrument can limit the throughput of this analysis and requires scrupulous clean up methodology. In developing methods for purification protocols for APIs, the use of ICP-MS is a limit on high throughput analysis of Pd levels.

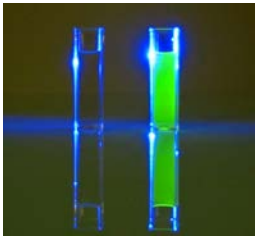
1. Heck RF. “Palladium Reagents in Organic Syntheses”. (New York, Academic Press, 1985).
2. Negishi, E and de Meijere, A. “Handbook of Organopalladium Chemistry for Organic Synthesis”. (New York, Wiley, 2002).
3. In “Environmental Health Criteria for Palladium”. International Programme on Chemical Safety, (WHO, 2002) QV 290.
4. In “Note for Guidance on Specification Limits for Residues of Metal Catalysts”. The European Agency for the Evaluation of Medicinal Products, Evaluation of Medicines for Human Use. (London December 17, 2002).
5. Rosso VW, Lust DA, Bernot PJ, Grosso JA, Modi SP, Rusowicz A, Sedergran TC, Simpson JH, Srivasava SK, Humora MJ, Anderson NG. “Removal of Palladium from Organic Reaction Mixtures by Trimercaptotriazine.” Org. Proc. Res. Dev. 1997; 1(4):311-314.
6. Ishihara K, Nakayama M, Kurihara H, Itoh A, Haraguchi H. “Removal of Palladium(II) from Aqueous and Organic Solutions by Polystyrene-bound Trimercaptotriazine.” Chem. Lett. 2000; 29(10):1218-1219.
7. Biotage Technical Note, “MP-TMT” website: www.biotage.com/DynPage.aspx?id=60724.
8. Prashad M, Bin H, Har D, Repic O, Blacklock TJ. “Efficient and Practical Syntheses of (R)-(5-Amino-2,3-dihydro-1H-inden-2-yl)-carbamic Acid Methyl Ester.” Adv. Synth. Catal. 2001; 343(5):461-472
9. Johnson Matthey Smopex™ website: smopex.com/page-view.php?page_id=160&parent_page_id=3.
10. Silicycle Technical document. “Application Note M3.0 Scavenging Palladium Catalysts.” website: http://ofsys.com/T/OFSYS/H/18011/Rtvca3/Appl_Note_M3.0Scavenging_Palladium_Catalysts.pdf.
11. Königsberger K, Chen G-P, Wu R, Girgis J, Prashad K, Repic O, Blacklock TJ. “An Improved and Efficient Process for the Production of Donepezil Hydrochloride: Substitution of Sodium Hydroxide for n-Butyl Lithium via Phase Transfer Catalysis.” Org. Proc. Res. Dev. 2003; 7(5):731-742.

ASSAY PRINCIPLE

The PdX® Palladium (Pd) Detection Kit is designed to allow the rapid determination of the relative amounts of Pd present in active pharmaceutical ingredient (API) scavenging steps. Please read the complete kit insert before performing this assay. The kit uses a patent-pending, exclusively licensed non-fluorescent detection molecule that, under reducing conditions, palladium cleaves to yield a brightly fluorescent product. See the reaction below:



Nonfluorescent Brightly Fluorescent



The relative concentrations of Pd in the sample from scavenging methods for removal of Pd is calculated after making a suitable correction for the dilution of the sample using software available with most plate readers.

In some Pd catalyst-API combinations the interaction between the Pd atom and the API molecule may be so strong that the Pd catalyst will not reduce the allyl moiety on the sensor (see page 14). In these cases we suggest microwave digestion of the API prior to running the fluorescent assay.

SUPPLIED COMPONENTS

Black 96 Well Plate
See: www.ArborAssays.com/resources/#general-info for plate dimension data.
One Plate Catalog Number X025-1EA

Palladium Standard
Palladium chloride at 2,000 nM in 1M hydrochloric acid. **Caution: Caustic.**
100 µL Catalog Number C016-100UL

PdX™ Palladium Detection Reagent
Palladium sensor solution in DMSO.
3 mL Catalog Number C017-3ML

Sodium Borohydride Stock Solution
A stock solution of sodium borohydride at 2.5M in 10M sodium hydroxide. **Caution: Caustic.**
400 µL Catalog Number X026-400UL

Borohydride Buffer
Borate buffer containing stabilizers.
5 mL Catalog Number X027-5ML

Sample Diluent
A Tris buffer containing DMSO and stabilizers.
60 mL Catalog Number X028-60ML

STORAGE INSTRUCTIONS

All components of this kit should be stored at room temperature until the expiration date of the kit.

OTHER MATERIALS REQUIRED

Borosilicate glass tubes or vials.

Optional: Microwave digestion apparatus with associated inert digestion cups. Nitric acid, hydrochloric acid and/or 30% hydrogen peroxide for digestion.

High quality N,N-dimethylformamide (DMF), such as catalog number 40228 from the Fluka division of Sigma-Aldrich, or similar.

Fluorescence 96 well plate reader capable of reading fluorescent emission at 520 nm, with excitation at 485 nm. Please contact your plate reader manufacturer for suitable filter sets.

Single channel and repeater pipettes.

Software for converting raw relative fluorescent unit (FLU) readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details. Set plate parameters for a 96-well Corning Costar 3650 plate. See www.ArborAssays.com/resources/#general-info for plate dimension data.

PRECAUTIONS

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The Palladium Standard stock is supplied in 1M hydrochloric acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

The sodium borohydride stock solution is supplied in 10M sodium hydroxide solution for stability purposes. **This solution is caustic - wear suitable laboratory clothing when handling this material.** The borohydride solution will generate hydrogen gas if diluted into acid solutions. Keep away from open flames.

Dimethyl sulfoxide is a powerful aprotic organic solvent that has been shown to enhance the rate of skin absorption of skin-permeable substances. Wear protective gloves when using the solvent especially when it contains dissolved chemicals.

SAMPLE TYPES

This assay is intended to be used to evaluate scavenger methods for removing palladium catalysts from API materials. It has been validated for certain Pd catalysts dissolved in a variety of solvent systems. Not all chemical structures and catalyst combinations have been tried. It is up to the end user to validate the use of the kit for their combinations in the solvent conditions they are using.

It is suggested that a reference level of the palladium content in the starting API prior to evaluation of scavenging methods be determined using ICP-MS or similar method and that this level is compared to the concentration determined using this kit as a baseline for determining the efficiency of Pd removal. Scavenger methods can then be assessed by evaluating decreases in fluorescent signal for each method. This kit is NOT intended to be used as a replacement to ICP-MS for final product validation.

SAMPLE PREPARATION

The assay is designed to assess scavenging methods with samples in a variety of solvent systems: Toluene, Ethanol, Acetonitrile, DMSO (**see note below**), DMF, N-Methyl Pyrrolidone (NMP) or dilute hydrochloric acid. Samples in, or which are soluble in, the water miscible solvents DMF, NMP, Acetonitrile, DMSO, Ethanol or acids (HCl, HNO_3, H_2SO_4) **must** be dissolved according to the following scheme. Water immiscible solvents should be handled as shown for toluene below:

Solvent System	API Concentration	1st Dilution required into DMF	2nd Dilution required into Sample Diluent
DMSO, DMF, NMP, MeCN	1 mg/mL	None	≥ 1:30
Ethanol	1 mg/mL	None	≥ 1:30
Acids (≤1M)	1 mg/mL	None	≥ 1:30
Toluene	20 mg/mL	≥ 1:20	≥ 1:30

Solutions of API's should be made in borosilicate containers. (If using ethanol or toluene, pre-fill the tip several times to ensure solvent vapor fills the space above the tip to ensure accurate delivery).

NOTE: DMSO can contain contaminants that interfere in the assay. Please contact us for further information.

Use all samples within 4 hours of dilution.

For any API-catalyst samples that fail to give linear dilutions and recovery, we suggest digesting the API in a chemical or microwave digestion instrument. Then dilute the digest in 0.1M nitric acid prior to dilution into Sample Diluent. Typical protocols have been published [12, 13].

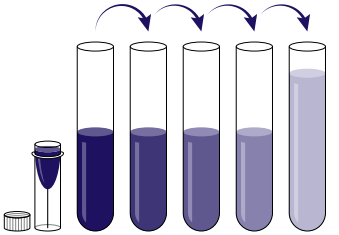
12. Niemelä, N, Kola, H, Eilola, K, Perämäki, P.j J. Pharma. Biomed. Anal, 2004: 35:433-439.
13. Wang, T, Walden S, Egan R., J. Pharma. Biomed. Anal, 1997: 15:593-599.

REAGENT PREPARATION

Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Standard Preparation

Label six borosilicate glass test tubes as #1 through #6. Briefly spin vial of standard in a microcentrifuge to ensure contents are at the bottom of the tube. Pipet 475 µL of Sample Diluent into tube #1 and 250 µL into tubes #2 to #6. Carefully add 25 µL of the supplied Palladium Standard solution to tube #1 and vortex completely. Take 250 µL of the 100 nM palladium standard solution in tube #1 and add it to tube #2 and vortex completely. Repeat these serial dilutions for tubes #3 through #6. The concentration of Pd in tubes 1 through 6 will be 100, 50, 25, 12.5, 6.25, and 3.125 nM.



Use all Standards within 2 hours of preparation.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Sample Diluent Vol (µL)	**475**	250	250	250	250	250
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5
Vol of Addition (µL)	**25**	250	250	250	250	250
Final Conc (nM)	100	50	25	12.5	6.25	3.125

Sodium Borohydride Reagent Preparation

Briefly spin vial of Sodium Borohydride in a microcentrifuge to ensure contents are at the bottom of the tube. Prepare the Sodium Borohydride Reagent by pipetting 2.5 mL of the Borohydride Buffer into a glass test tube and removing 90 µL. Carefully add 90 µL of the Sodium Borohydride Stock solution to the tube and vortex.

This volume of Borohydride is sufficient for the complete plate - adjust for partial plate use. Use Reagent within 2 hours of dilution.

ASSAY PROTOCOL

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine Pd concentrations.

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification. Set plate parameters for a 96-well Corning Costar 3650 plate.
   See: www.ArborAssays.com/resources/#general-info for plate dimension data.
2. Pipet 100 µL of samples or standards into wells in the plate. Pipet 100 µL of Sample Diluent into the wells for a zero standard.
3. Add 25 µL of the PdX™ Palladium Detection Reagent to each well, using a repeater pipet.
4. Add 25 µL of the Sodium Borohydride Reagent solution to each well using a repeater pipet.
5. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
6. Incubate for 30 minutes at room temperature.
7. Read the fluorescent signal from each well in a plate reader capable of reading the fluorescent emission at 520 nm with excitation at 485 nm. Please contact your plate reader manufacturer for suitable filter sets.
8. Use the plate reader's built-in 4PLC software capabilities to calculate Pd concentration for each sample.

CALCULATION OF RESULTS

Average the duplicate FLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean FLUs for the zero standard. The sample concentrations obtained should be multiplied by the dilution factor to obtain neat sample values.

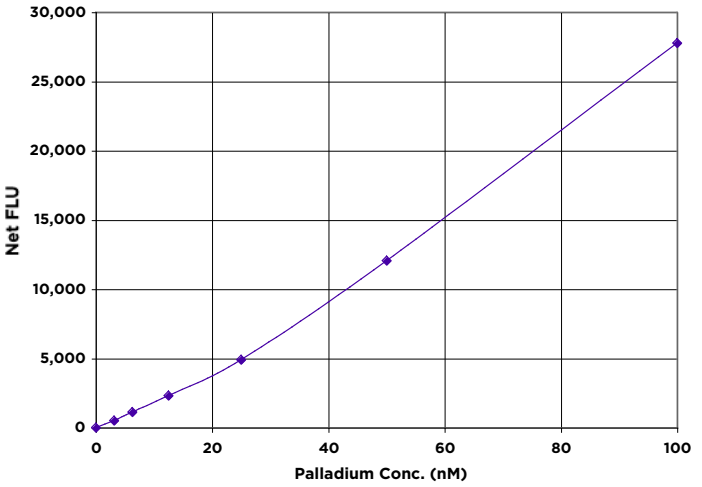
Or use the online tool from MyAssays to calculate the data:
www.myassays.com/arbor-assays-palladium-pdx-api-fluorescent-kit.assay

TYPICAL DATA

Sample	Mean FLU	Net FLU	Pd Conc. (nM)
Zero	241	-	0
Standard 1	28,009	27,769	100
Standard 2	12,299	12,058	50
Standard 3	5,147	4,907	25
Standard 4	2,559	2,319	12.5
Standard 5	1,370	1,129	6.25
Standard 6	746	506	3.125
Sample 1	19,257	19,016	72.6
Sample 2	3,183	2,943	16.1

Always run your own standard curve for calculation of results. Do not use this data.

Typical Standard Curve

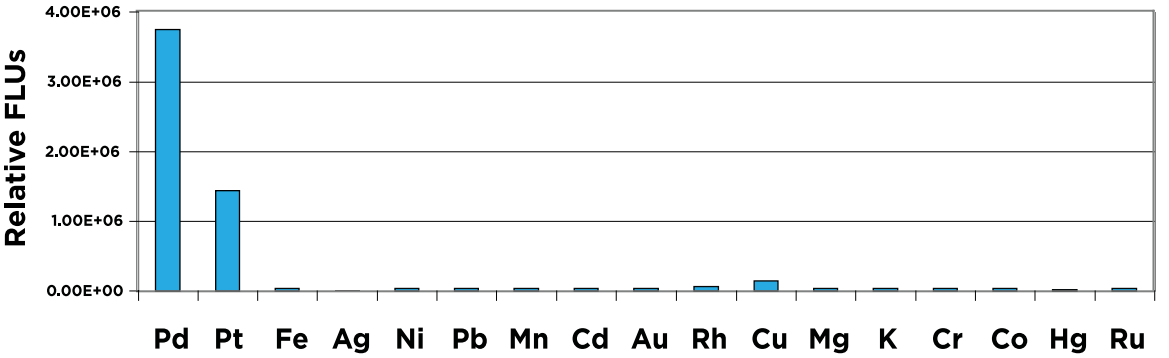


Example

An API sample at 1 mg/mL in DMF with 100 ppm Pd contamination would have a Pd concentration of 100 ng/mL which is equivalent to 939.7 nM. After being diluted 1:30 in Sample Diluent, per directions on page 7, this sample would read at 31.3 nM.

METAL SPECIFICITY

The PdX™ Palladium Detection Reagent is selective for palladium and platinum over other metal ions. In the experiment below, other metal ions were run at 10 times the concentration of the palladium and platinum ions.



Graph Caption:
$PdCl_2$ and $PtCl_2$ tested at 1 µM.

$FeCl_3$ = $AgNO_3$ = $NiCl_2$ = $Pb(NO_3)_2$ = $MnCl_2$ = $CdCl_2$ = $AuCl_3$ = $RhCl(PPh_3)_3$ = $CuCl_2$ = $MgSO_4$ = KCl = $CrCl_3$ = $CoCl_2$ = $HgCl_2$ = $RuCl_3$ all tested at 10 µM.

COLORED INTERFERENTS

To assess the effect of colored API's on the measured palladium concentration in the fluorescent assay we chose three FD&C food color preparations (Red 40 & 3 for red, Yellow 5 for yellow, and Blue 1 for blue) representing red, yellow and blue colored materials. These were added at 1:800 and 1:3200 dilutions to palladium catalyst solutions in Sample Diluent and compared to the same concentrations of palladium catalyst solutions without dyes and read in the assay. The data are shown below:

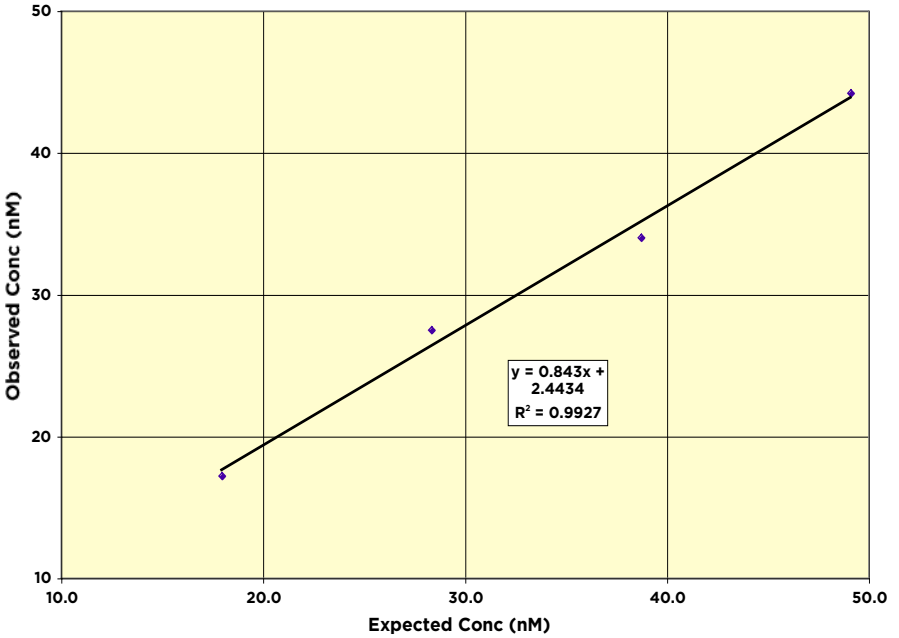
	Dye Dilution = 1:800		Dye Dilution = 1:3,200	
Sample	**Pd Conc. (nM)**	**% Interference**	**Pd Conc. (nM)**	**% Interference**
Sample Pd	76.2	- - -	76.2	- - -
Pd w/ Red FC	61.4	19.4	70.9	7.0
Pd w/ Yellow FC	63.4	16.8	79.7	-4.6
Pd w/ Blue FC	66.5	12.7	76.9	-0.9

VALIDATION DATA

Linearity

Linearity was determined by taking two palladium catalyst samples in toluene diluted 1:20 in DMSO and then diluted in Sample Diluent, one, Bis(dibenzylideneacetone) palladium(0), with a low diluted Pd level of 7.6 nM and one, 2-(2'-Di-tert-butylphosphine)biphenylpalladium(II) acetate, with a higher diluted level of 59.5 nM, and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

Low Pd Sample	High Pd Sample	Expected Pd Conc. (nM)	Observed Pd Conc. (nM)	% Recovery
80%	20%	18.0	17.2	95.7
60%	40%	28.4	27.5	97.0
40%	60%	38.7	34.0	87.8
20%	80%	49.1	44.2	90.0
			Mean Recovery	**92.6%**



Intra Assay Precision

Three different palladium catalyst samples were diluted with Sample Diluent and run in replicates of 20 in an assay. The mean and precision of the calculated Pd concentrations were:

Sample	Pd Concentration (nM)	%CV
1	16.5	10.4
2	48.3	9.9
3	76.4	14.9

Inter Assay Precision

Three different palladium catalyst samples were diluted with Sample Diluent and run in duplicates in twenty assays run over two days by four operators. The mean and precision of the calculated Pd concentrations were:

Sample	Pd Concentration (nM)	%CV
1	16.6	4.1
2	56.0	5.2
3	83.0	5.5

CATALYST RECOVERY

Five Pd catalysts were dissolved in DMSO to a catalyst concentration of 1mg/mL and the concentration of Pd was calculated. Each catalyst was diluted to working concentrations of 20 and 5 nM Pd in Sample Diluent and run in the assay alongside $PdCl_2$ control dilutions of the same working concentrations. The obtained values were evaluated for linearity off the lowest concentration (5 nM) and for percent recovery as compared to the control values.

	Nominal Pd Conc (nM)	Observed Conc (nM)	Linearity	Obs.Cntrl Conc (nM)	%Recovery
Catalyst 1	20	16.81	94.1%	19.54	86.0
Catalyst 2	20	16.17	99.8%	---	80.9
Catalyst 3	20	16.67	107.4%	18.56	89.8
Catalyst 4	20	13.80	110.8%	19.97	69.1
Catalyst 5	20	17.87	91.8%	19.39	92.2

Palladium catalysts were:
Catalyst 1 = Allylpalladium(II) chloride dimer
Catalyst 2 = Bis-(dibenzylideneacetone)palladium(0)
Catalyst 3 = Bis(triphenylphosphine)palladium(II) dichloride
Catalyst 4 = 2-(2'-Di-tert-butylphosphine) biphenylpalladium(II) acetate
Catalyst 5 = Palladium(II) acetate

API INTERACTION TESTS

We obtained 5 chemical compounds that bind extremely tightly to palladium catalysts and could be representative of structures found in APIs. These 5 chemical entities were A, 2-methyl-2-oxazoline; B, thiazole; C, 4,4'-diphenyl-2,2'-dipyridyl; D, 8-aminoquinoline; and E, thiazolidine-2-carboxylic acid. The structures are shown below.

Each of the 5 compounds (A-E) were dissolved in DMSO. Each compound was then spiked with the Pd catalysts tested on page 13 and the palladium standard, $PdCl_2$ to concentrations equivalent to 10 ppm Pd contamination. Each compound without added palladium catalysts was run as compound controls. Each catalyst spiked into DMSO without a compound was run as catalyst controls.

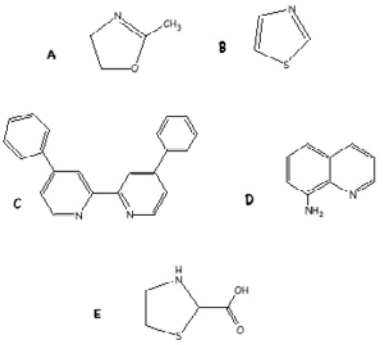
Multiple dilutions of each of the solutions were made into Sample Diluent and run in the assay. The obtained values for each compound + catalyst, as well each compound control, were evaluated for recovery against the catalyst control values. No signal was detected for compound only controls.

	Average Recoveries				
Catalyst or Standard	Compound A 50 mg/mL	Compound B 50 mg/mL	Compound C 5 mg/mL	Compound D 50 mg/mL	Compound E 5 mg/mL
1 ≃ 10 ppm	101.6%	78.7%	54.6%	61.3%	ND
2 ≃ 10 ppm	93.8%	67.6%	36.7%	73.4%	ND
3 ≃ 10 ppm	155.8%	58.8%	ND	90.2%	ND
4 ≃ 10 ppm	103.6%	60.9%	ND	48.7%	75.6%
5 ≃ 10 ppm	99.5%	67.4%	ND	75.0%	ND
$PdCl_2$ Standard	107.4%	81.2%	ND	89.1%	ND

ND = Not detectable.

Compound Structures

A = 2-methyl-2-oxazoline
B = thiazole
C = 4,4'-diphenyl-2,2'-dipyridyl
D = 8-aminoquinoline
E = thiazolidine-2-carboxylic acid



For Compound A adequate recoveries were made with each of the 5 catalysts tested. Compounds B and D showed lower than ideal recovery (48.8-90.2%). Compounds C and E showed low recovery or no recovery of added catalyst as Pd.

For compounds such as C and E where simple dilution does not yield suitable Pd results, we suggest chemical or microwave digestion in nitric acid to ensure correct detection of palladium catalyst contamination concentrations.

LIMITED WARRANTY

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

CONTACT INFORMATION

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OFFICIAL SUPPLIER TO ISWE

Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with EIA kits for wildlife conservation research.

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