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Electrophoretic monitoring of pollutants: Effect of cations and organic compounds on protein interactions monitored by native gel electrophoresis

We describe how the interaction between actin and its protein ligands can be used to evaluate the presence of certain metal (Cd, Cu, Hg, Zn) ions and organic compounds (2,4-dioxin or Picloram) which are common components of environmental pollution. The assay detects the high-affinity binding of actin to actin-binding proteins (ABPs), cofilin or DNase I. The actin-ABP complex was analyzed using native polyacrylamide gel electrophoresis and quantified by scanning densitometry. These proteins are widely distributed in animals and plant cells. The assay involves allowing the proteins to form an actin-ABP complex into which increasing amounts of pollutants are titrated. Thus, the assay directly tests for inhibition of protein-protein interaction. It is sensitive to common pollutants using concentration ranges over which they are known to exert a biological toxicity. A convenient feature of the assay is the fact that all the proteins can be stored in freeze-dried form, and can be purchased commercially. We suggest that if this molecular assay is sensitive to a wide range of environmental pollutants, it could be used as a rapid and convenient assay of the environment in combination with currently available tests.

Keywords: Actin / Actin-binding proteins / Cofilin / Native gel electrophoresis / Heavy metal ions

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1 Introduction

The reliable analysis of toxicants present in aqueous samples is an important aspect of environmental monitoring of waterways. Bioassays of liquid leachate samples from toxic sites should aim to involve as wide a range of assay methods and systems as possible, thus improving the reliability and their biological relevance. Bioassays can be classified into four categories: (i) whole metazoan animal assays such as assays of the rodents, fresh water copepods (crustacean) or the marine echinoderm assays [1], or plant-based assays; (ii) single-cell assays such as those based on light emitted from the luminescent marine bacterium, *Photobacterium phosphoreum*, commonly known as the Microtox test [2], and other single-cell assays such as the lymphocyte assay [3]; (iii) subcellular assays using either beef heart whole mitochondria or sub-mitochondrial particles in which membrane-based enzyme systems are used to assay the toxicity of sam-

ples [4]; and finally, (iv) we suggest that a new type of assay be considered, namely a molecular bioassay in which the formation of a complex between two naturally occurring protein molecules forms the basis for sensing the presence of toxic ligands. The range of test currently approved by the Environmental Protection Agency (EPA) in the United States is limited to only the first of these categories. For example, the US EPA has nominated eight taxa for freshwater toxicity testing and a similar number for the testing of marine toxins. Rather than argue the merits, or otherwise, of a particular assay we suggest that the scope or breadth of these tests be expanded so that at least one assay from each category be included in a suite of tests. Such a widening of the analysis base would provide greater biological diversity and therefore a more complete picture of the toxicity of a sample. We will argue that the results of tests covering categories (iii) and (iv) are applicable to a wide range of eukaryotes. However, the toxicity will depend on the pharmacokinetics and pharmacodynamics which determine whether the toxins actually reach their final destinations in cells (*i.e.* mitochondria and cytoskeleton).

Actin is a natural and abundant protein. It is responsible for a wide range of cellular activities including cytoplasmic streaming, cell transport, and cell division. Actin occurs in significant quantities in virtually all eukaryotic cells, animal

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Abbreviations: **ABP**, actin-binding protein; **BHM**, beef heart mitochondria; **2,4-D**, 2,4-dioxin; **SMP**, submitochondrial particles

or plant. It comprises more than 12–15% of the total proteins in motile cells or cells which exhibit cytoplasmic streaming, and accounts for about 30% of muscle cell proteins. Even in nonmotile cells such as red blood cells, actin comprises about 1–2% of the cytoskeletal proteins. Actin is the major component of the microfilaments of cells, and it is one of the most widely cited proteins in the literature (see [5–7] for major reviews of the structure and function of actin in muscle and nonmuscle contractile systems). Actin, DNase I and cofilin are available commercially, which means that the test can be reproduced without the need to isolate and purify these two proteins. *In vivo*, the state of assembly/disassembly of actin is regulated by one of a number of actin-binding proteins (ABPs), the most abundant of which is cofilin [8]. Actin and cofilin form a high-affinity (k_D , approx. 10^{-8} M^{-1}) complex which has been well characterised *in vitro* [9–11]. Actin also can form a high-affinity complex with another ABP, DNase I [12].

2 Materials and methods

2.1 Actin preparation

Actin was prepared from an acetone-dried powder of rabbit skeletal muscle according to the method of Spudich and Watt [13] with slight modifications as described in Barden and dos Remedios [14]. A typical yield of actin monomers was approximately 50 mL of 60–90 μM (2–4 mg/mL) protein in a buffer (2 mM Tris, pH 8.0, 0.2 mM ATP, 0.1 mM CaCl_2 , 1 mM dithiothreitol). G-actin was snap-frozen in liquid nitrogen, freeze-dried and then stored at -20°C . Monomeric actin concentration was determined from its OD at 290 nm, where $E_{0.1\%} = 0.63$ [14].

2.2 Expression and purification of chicken cofilin

Recombinant cofilin (M_r , 18.7 kD) cDNA was expressed as a glutathione-S-transferase (GST) fusion protein. Briefly, *Escherichia coli* transformants were grown at 37°C in LB medium containing 60 $\mu\text{g/mL}$ ampicillin and induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when the OD_{600} was 0.6. After 3 h the cells were harvested by centrifugation and sonicated in lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, 0.5 mM PMSF, 1% Triton X-100, pH 8.0) or the cells were disrupted using a French press. After centrifugation, the supernatant was applied to a glutathione Sepharose 4B column (Pharmacia, Piscataway, NJ, USA) equilibrated with PBS, 0.5 mM PMSF, 1 mM EDTA, pH 7.3. The column was washed with 2– column volumes of PBS and then three column volumes of thrombin buffer (50 mM

Tris, 2.5 mM CaCl_2 , 50 mM NaCl, pH 8.0). The matrix was suspended in 100 units of thrombin/L of culture and incubated at 37°C for 1 h with the matrix being mixed at 15 min intervals. Cofilin was eluted with 2–3 column volumes of thrombin buffer. The purified cofilin was dialysed against water, snap-frozen in liquid nitrogen and freeze-dried for future use. The cofilin concentration was estimated by using: $E_{0.1\%} \text{ at } 280 \text{ nm} = 0.93$ [15].

2.3 DNase I

DNase I was purchased from either Worthington Biochemicals (Freehold, NY, USA) or from Calbiochem (La Jolla, CA, USA) and was used without further purification.

2.4 Native gel electrophoresis

Native and SDS-PAGE were performed with a Bio-Rad Mini Protean II system (Richmond, CA, USA) using a discontinuous Tris-glycine buffer system [16]. The stacking gel contained 5% acrylamide in 80 mM Tris-glycine (pH 6.8) and the separating gel was either 10 or 12% acrylamide in the same buffer, adjusted to pH 8.6 at 125 V for 90 min. Samples were run at $0-4^\circ\text{C}$ by packing the gel apparatus in ice in native sample buffer (62.5 mM Tris, 10% glycerol, 0.1% bromophenol blue, pH 6.8). Protein bands were visualised by staining with Coomassie Brilliant Blue R-250 destained in 40% methanol, 7% acetic acid overnight.

2.5 Heavy metal ions and organic compounds

Metal ions were analytical reagent grade and were dissolved as chlorides. 2,4-Dioxin (2,4-D) and Picloram were kindly provided by Dr. J. Pollak. These inorganic ions and organic compounds were tested over a range of concentrations to examine their ability to dissociate the actin-cofilin complex. Their relative dissociating effect was evaluated by determining the concentration required to dissociate the complex by 50% (E_{50}). DNase I was purchased from Worthington Biochemicals.

3 Results

3.1 Electrophoresis of complexes of actin-cofilin or actin-DNase I in native gels

Combinations of actin, cofilin and DNase I were mixed at equimolar concentrations ($\sim 20-25 \mu\text{M}$) in a Eppendorf tube and incubated for 15 min at 22°C . Actin, cofilin, DNase I and their complexes were then separated on a 10% native gel as illustrated in Fig. 1. Since the separations in this gel were achieved under non-denaturing conditions, the proteins do not migrate according to their ap-

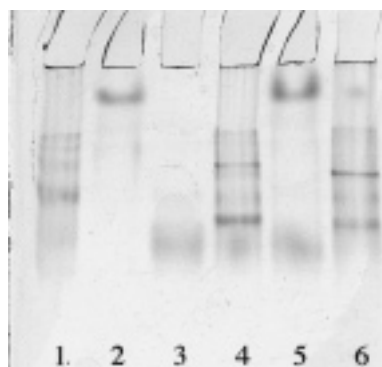


Figure 1. A 10% PAGE native gel illustrating the interaction of actin with cofilin or DNase I (Worthington). Lane 1, monomeric actin (9 μg load); 2, cofilin (4 μg); 3, DNase I (8 μg); 4, actin (9 μg load) mixed with DNase I (8 μg); 5, cofilin (4 μg) mixed with DNase I (8 μg) in the absence of actin; 6, actin (9 μg load) mixed with cofilin (4 μg). The gel was stained with Coomassie blue.

parent molecular weights as they do in SDS-PAGE gels. Instead, the rate of migration is determined by the ratio of charge to volume. Actin (M_r 43 kDa) has a net negative charge. Under conditions of low ionic strength, monomeric actin is seen (lane 1) as a major monomer band migrating ahead of lesser amounts of dimer and higher oligomers up to the interface between the stacking (5%) and running (10%) gels. Cofilin is about half the molecular mass (18.7 kDa) of actin, but because of its net positive charge at the pH conditions in the native running gel (pH 8.6), it remains in the stacking gel (lane 2). DNase I (M_r 36 kDa) migrates as an indistinct band (lane 3) moving ahead of the actin monomers. When cofilin and DNase I are mixed in equimolar amounts, there is no evidence that the two form a complex (lane 5).

An equimolar complex of actin with either DNase I or cofilin produces two bands which are more sharply defined than any of the proteins alone. A mixture of actin with DNase I (lane 4) results in two sharp bands, a lower band which migrates near the actin monomer and free DNase bands (but is not identical to either), and a slightly less dense upper band. A similar, but nonidentical pair of bands, is seen when actin is complexed to cofilin (lane 6). Note the reduction in densities of the unbound DNase I and cofilin bands in lanes 4 and 6, respectively. These new actin-ABP complexes are the focus of the assays described below.

3.2 Heavy metal cations alter the ability of actin to interact with cofilin

Four heavy metal ions (Cd, Cu, Hg and Zn) were examined over the concentration range of 1–1000 $\mu\text{g/L}$. These ions were selected because they are commonly present

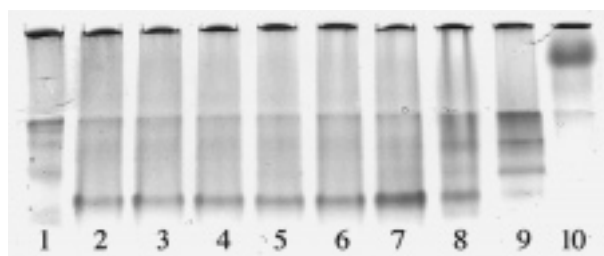


Figure 2. A 12% PAGE native gel illustrating the effects of Cd and Hg on the actin-cofilin complex. Lane 1, monomeric actin (13.5 μg); 2, actin-cofilin (13.5 μg and 8 μg respectively); 3–5, the actin-cofilin complex plus 1, 5 and 10 $\mu\text{g/L}$ CdCl₂, respectively; 6–9, the actin-cofilin complex plus 1, 5, 10 and 20 $\mu\text{g/L}$ HgCl₂ respectively; 10, cofilin (8 μg) only.

in polluted water samples. The effects of adding increasing concentrations of Cd chloride (lanes 3–5) and Hg acetate (lanes 6–9) to the actin-cofilin complex are illustrated in native 12% acrylamide gel shown in Fig. 2. Untreated actin is shown in lane 1 and the actin-cofilin complex (25 μM) with no added cation is the prominent band in lane 2. Cofilin alone is shown in lane 10. The volume densities of all bands (spot area \times the integral of the stain density) were determined by densitometry. The ability of Cd (1, 5 and 10 $\mu\text{g/L}$) to decrease the density of the actin-cofilin complex is very weak (Fig. 1, lanes 3–5, respectively).

Table 1. Integrals of volume density of the actin-cofilin band from the native PAGE gel illustrated in Fig. 2 (for Hg) and for Cu (gel not shown).

Sample	Ligand concentration ($\mu\text{g/L}$)	Volume density (arbitrary units)	E_{50} $\mu\text{g/L}$
Actin-cofilin only		391	
Actin-cofilin, Hg	1	655	
Actin-cofilin, Hg	5	920	
Actin-cofilin, Hg	10	442	
Actin-cofilin, Hg	20	154	40
Actin-cofilin, only	–	303	
Actin-cofilin, Cu	250	280	
Actin-cofilin, Cu	500	227	
Actin-cofilin, Cu	1000	49	~600
Actin-cofilin, only	–	490	
Actin-cofilin, Picloram	500	407	
Actin-cofilin, Picloram	1500–2000	742	1500–3000
Actin-cofilin, 2,4-D	4000–5000	68	~2000

The amounts of added metal ions ($\mu\text{g/L}$) did not include the mass of the counter ions (chlorides or acetate). Effective concentrations (E_{50} $\mu\text{g/L}$) were determined from multiple gels and are precise to $\pm 10\%$.

These Cd concentrations were selected to compare with the effects of Hg (below), but a much wider concentration range (not shown) was needed for the calculation of an E_{50} value of 800–1200 $\mu\text{g}/\text{mL}$ (Table 2). The principal effect of Cd was a decrease in the density of the actin-cofilin band.

In contrast, the complex is most sensitive to progressive addition of 1–20 $\mu\text{g}/\text{L}$ Hg (added as the acetate). The volume densities of the actin-cofilin bands are listed in Table 1. Addition of 1 and 5 $\mu\text{g}/\text{L}$ results in a progressive increase in the density of the actin-cofilin complex (lanes 6, 7), whereas 10 $\mu\text{g}/\text{L}$ reduces the density of this band to levels comparable with the control (lane 2) with the simultaneous appearance of a series of slower bands (lane 8). At 20 $\mu\text{g}/\text{L}$ (lane 9) this trend is accentuated with the almost complete loss of the native actin-cofilin band. The absence of unbound cofilin near the top of the gel suggests that native cofilin is not dissociated. From this and other determinations (not shown) we estimate that the E_{50} for Hg for the disappearance of the actin-cofilin band is approximately 20–40 $\mu\text{g}/\text{L}$ using 25 μM actin-cofilin complex. This binding ratio suggests that one Hg^{2+} binds to a highly reactive cysteinyl residue in the actin-cofilin complex.

Cu^{2+} does not noticeably affect the actin-cofilin complex until it reaches a concentration of approximately 200 $\mu\text{g}/\text{L}$. At higher Cu^{2+} concentrations, formation of the actin-cofilin complex is progressively inhibited and the proteins seem to disperse evenly along the gel lane. The 50% effective dose (E_{50}) for Cu^{2+} is approximately 400–600 $\mu\text{g}/\text{L}$. Cu^{2+} can bind to a number of amino acid side chains, for example, it is known to bind to Phe-375 of the actin sequence [17]. In fact, Cu Binding has been used as a quantitative test for proteins [18]. Judging by the E_{50} values, disruption of the actin-cofilin complex is about an order of magnitude more sensitive to Hg^{2+} than to Cu^{2+} . We tested the effects of adding ZnCl_2 using the concentration in the range 250–2500 $\mu\text{g}/\text{L}$. At these relatively high concentrations, it decreased the density of the actin-cofilin complex. The E_{50} value (900–1000 $\mu\text{g}/\text{L}$) was significantly higher than either Hg or Cu. Thus, increasing sensitivity of the actin-cofilin band to these transition metal ions was achieved in the order: $\text{Hg} \gg \text{Cu} > \text{Cd} \geq \text{Zn}$.

3.3 Heavy metal cations alter the ability of actin to interact with DNase I

We investigated whether heavy metal ions interfered with the interaction of actin with other ABPs. Like cofilin, DNase I binds to actin monomers with high affinity. Figure 3 is essentially identical to Fig. 2 except that DNase I

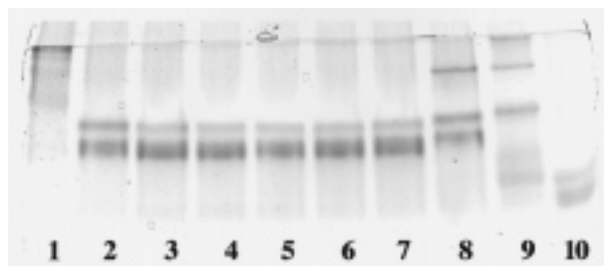


Figure 3. A 12% native PAGE gel illustrating the effects of Cd and Hg on the actin-DNase I (Calbiochem) complex. Lane 1, monomeric actin (13.5 μg); 2, actin-DNase I (13.5 μg and 11 μg , respectively); 3–5, the actin-DNase I complex plus 1, 5 and 10 $\mu\text{g}/\text{L}$ CdCl_2 , respectively; 6–9; the actin-DNase I complex plus 1, 5, 10 and 20 $\mu\text{g}/\text{L}$ HgCl_2 , respectively; 10, DNase I (11 μg) only.

Table 2. Effective (E_{50}) concentrations

Metal cation	Actin-cofilin ($\mu\text{g}/\text{L}$)	SMP ($\mu\text{g}/\text{L}$)	BHM ($\mu\text{g}/\text{L}$)	Fish ($\mu\text{g}/\text{L}$) (at 96 h)	Microtox ($\mu\text{g}/\text{L}$)
Hg^{2+}	20–40	130	126	170	59
Cu^{2+}	400–600	300	93	530	9300
Cd^{2+}	800–1200	520	158	630	41400
Zn^{2+}	900–1000	1700	80	2990	33000

E_{50} concentrations were determined by: (i) analysis of native PAGE gels of actin-cofilin; (ii) submitochondrial particle (SMP) assay; (iii) beef heart mitochondria (BHM) assay; (iv) whole organism (Fish) assay; and (v) mean lethal concentrations (at 96 h) for *Photobacterium phosphoreum*, the Microtox assay. The amounts of added metal ions ($\mu\text{g}/\text{L}$) did not include the mass of the counter ions (chlorides or acetate). Data cited from Read *et al.* [4].

replaced cofilin. Note that the Calbiochem DNase I (far right-hand lane) migrates as a doublet band ahead of the actin-DNase I complex. Cd (1, 5 and 10 $\mu\text{g}/\text{L}$) has only a weak effect (E_{50}) on the actin-DNase I complex compared to Hg. We did not undertake a systematic analysis of the effects of Cu or Zn on the actin-DNase I complex.

3.4 Organic pollutants alter the ability of actin to interact with cofilin

We examined the effects of Picloram as well as both the acid and salt forms of 2,4-D. The effect of 2,4-D salt was too weak to determine a value for E_{50} . We were unable to determine the E_{50} since it did not have an apparent dissociation effect on the actin-cofilin complex. The acid form of 2,4-D was slightly more effective in producing a gradual increase in the density of the actin-cofilin band with 500, 1000 and 2500 $\mu\text{g}/\text{L}$ (Fig. 4, lanes 3–5, respectively). In contrast, Picloram did interfere (500, 1000 and 2500 $\mu\text{g}/\text{L}$, Fig. 4, lanes 6–8) with the actin-cofilin complex and its effect resembled Hg, namely it first induced an intensifica-

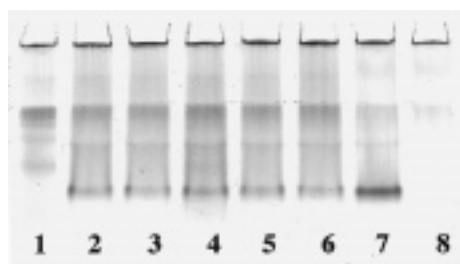


Figure 4. A 12% native PAGE gel stained with Coomassie (see Section 2.4). Samples are: lane 1, monomeric actin; 2, actin with cofilin only (9 µg and 4 µg, respectively); 3–5, actin with cofilin (as in lane 2) plus 500, 1000, and 2500 µg/L, respectively, of the acid form of 2,4-D; 6–9, actin with cofilin plus 500, 1000, and 2500 µg/L, respectively, of Picloram; 10, cofilin only.

tion of the actin-cofilin complex (lane 7) and then at higher concentrations it dissociated the complex (lane 8). We estimate the E_{50} to be ~1500/–;3000 µg/L. We also examined the effects of dimethyl formamide on the stability of the actin complex. The E_{50} value for DMF was in excess of 4000 µg/L (data not shown).

4 Discussion

Heavy metal cations are commonly assessed by chemical (atomic absorption) analysis but this form of assay tells us nothing about their ionised state, even though most biologically active heavy metals are ionised. Furthermore, although chemical analysis may be quantitatively highly precise, it is not informative about the biological activity of the analyte. Bioassays are more complex, they often require substantial infrastructure (*e.g.* holding tanks for breeding live organisms), and they can be labour-intensive. Assays of marine samples can be achieved using an echinoderm toxicity/development assay [1], whereas fresh water (aquatic) assays are commonly done using copepods, or small teleost species. Single cell assays have been developed which employ prokaryotic (Microtox) populations [2], and more recently a T-cell lymphocyte assay has been developed [19]. The test outlined in this report is based on the interactions between two well-defined proteins; it is therefore simpler to interpret than tests based on whole beef heart mitochondria (BHM) or their derivatives, submitochondrial particles (SMP assay), and it is substantially different to systems which use whole cells or organisms.

These whole-organism tests have a well-established place in biotoxicity assays and, in fact, are the only test approved by the US Food and Drug Administration (FDA). It is reasonable to conclude that if the organism

dies, the pollutant must be toxic. Newer assays based on single-cell or even subcellular assays are currently under consideration. We believe that a diversity of bioassays should be developed to monitor samples of possibly polluted waterways. Subcellular particles, such as mitochondria, can be directly used or they can be mechanically disrupted, turning the mitochondrial membranes inside out (submitochondrial particles). The activities of exposed inner membrane-bound enzymes can then be quantified spectrophotometrically [4]. The nature of these assays is more complex than the actin-cofilin/DNase I assay, but both are reasonably well defined at the molecular level.

Table 2 compares the sensitivities of the actin-cofilin test with: the SMP test; frozen-thawed whole BHM; the flat-head, minnow or bluegill (Fish) assay (based on a lethal dose over a period of 96 h); or the photoluminescence test using the *Photobacterium phosphoreum* (Microtox) test. These data are reproduced from [4]. We show that the actin-cofilin molecular interaction assay is more sensitive to Hg than the SMP assay (or any other test) and is slightly less sensitive to Cd and Zn. However, the order of sensitivity of the actin-ABP test for all four divalent cations is nearly the same ($Hg \gg Cu > Cd \geq Zn$) for the actin-ABP test as it is for all other listed assays. The hypersensitivity of the actin-cofilin assay to Hg may well be due to actin, which has a structurally important cysteinyl. Actin contains four Cys residues. The one near its *N*-terminus (Cys374) can be chemically modified with little affect on its function. A second reactive Cys is located near its *N*-terminus (Cys-10) and its modification probably distorts the actin conformation [20]. The similarity of data for cofilin and DNase I suggests that the sensitivity of the complex may not be due to cofilin, or DNase I. Cys-10 lies in a sensitive region of subdomain 1 of actin, a part of the structure known to be influenced by covalent modifications and by changes in actin conformation [21].

The current data suggest that assays based on whole organism and subcellular particles could be supplemented with a molecular assay based on protein-protein interactions. A suitable protein-protein ligand pair might be actin and cofilin, but any pair of tightly binding macromolecules could form the basis for such a test. Furthermore, the actin-cofilin assay is more sensitive to all four heavy metal ions than the Microtox assay [4]. Our molecular assay has several advantages: (i) it is rapid; for example, native gel electrophoresis can be completed and the gel stained in a matter of hours of adding an unknown sample; (ii) a 15 lane gel can test multiple samples; (iii) actin, cofilin and DNase I can be freeze-dried and, in this form, they are stable over long periods (*e.g.*, up to one year at -20°C) and can be stored at room temperature; and finally (iv) the molecular assay is based on

biologically relevant proteins which are present in significant amounts in all eukaryotic (plant or animal) cells. Others [22] have shown that the affinity of cofilin for actin is only slightly reduced in the presence of 140 mM NaCl, thus our assay is not likely to be affected by salt.

Now that the basis for this molecular assay has been established, we plan to extend its use to a wide range of metal ions, solvents, insecticides, herbicides and pharmaceutical compounds [4]. These results will be necessary prerequisites to establishing the use of an actin-ABP assay as a member of a suite of tests of polluted environmental samples, particularly Hg, which is used in the silver and gold mining industry [23]. In considering and testing the toxicity of these and other pollutants it is important to consider other factors (such as components in the solvent system) which might significantly alter their biological toxicity [24].

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