

HazardScreen

Information Memorandum

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Prepared by *Strategic Ventures*
Tel: 612 9234 3888 Fax: 612 9234 3800
e-mail: mail@strategic.net.au
www.strategic.net.au

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Overview

Water is the single most important resource available to people, livestock, and vegetable life. Access to a safe, drinkable water supply is an issue that grows increasingly important as both the population and industry grow at alarming rates, putting a strain on available resources.

This is most prevalent in developing nations where extraordinary population growth and lack of environmental legislation has substantially degraded drinkable water supplies.

HazardScreen has the potential to place in the hands of any interested party the power to identify toxic water.

Company

HazardScreen is an entity created to develop and commercialize the IP developed by Professor Cris dos Remedios, Professor Roger Cooke and Mr Murat Kekic. *Strategic Ventures* owns 51% of equity in *HazardScreen* with the remainder being controlled by the developers of the IP.

Market

We are confident that the entry cost, usability and functionality of our technology will allow for the development of products that will substantially compete in and extend existing lucrative markets.

Also, we believe that factors such as the growing concern about environmental issues, increased awareness about what we consume, increased regulatory and moral concerns for industry, and the degradation of water supplies in populous areas will lead to an increased demand for such products.

Technology

HazardScreen has developed a test for the detection of toxicants in water based on the observation of the inhibiting effects of pollutants on the interaction between the binding partnership of two molecules.

To date, toxicity-testing methods have been cumbersome and require a power source, or significant infrastructure, expense and skilled personnel.

The aim has been to develop a quick, simple, portable, accurate and inexpensive test for environmental pollution of water samples.

Research

The goal of the research program has been to develop methods easily adaptable to a range of in-the-field water toxicity tests.

To date, research has focused on two embodiments, one based on protein-protein interactions; the second involving the use of molecules that fluoresce when they bind to DNA.

Company

HazardScreen is an entity created to develop and commercialize the IP developed by Professor Cris dos Remedios, Professor Roger Cooke and Mr Murat Kekic.

Strategic Ventures owns 51% of equity in *HazardScreen* with the remainder being controlled by the developers of the IP.

Global patents have been applied for in the name of the inventors. *HazardScreen* obtains the control of the IP contained in the patents by way of a license agreement.

Research team

The IP was developed by Professor Cris dos Remedios (Professor of Anatomy & Biophysics, Muscle Research Unit, Institute for Biomedical Research, The University of Sydney) in collaboration with Professor Roger Cooke (University of California, San Francisco) and Mr Murat Kekic (PhD student, University of Sydney).

Both Professor dos Remedios and Professor Cooke have worked in the field of contractile protein chemistry for over thirty years. These two scientists have published about 200 research papers in this and related fields.

Mr Murat Kekic completed an M.Sc at The University of Sydney under the supervision of Professor C.G. dos Remedios. He then enrolled as a PhD student, at the same University funded by a scholarship from the Faculty of Medicine at this University.

The Chief Investigator, Professor dos Remedios has published 110 papers (refer to list of publications attached) in International peer-reviewed journals including one international patent (not related to *HazardScreen*). They demonstrate relevant expertise in the preparation and experimentation with actin and actin-binding proteins and with heavy metal ions, and the production of anti-actin monoclonal antibodies.

Market

Environmental, and in particular water testing is the most obvious and readily exploitable market for products using *HazardScreen* technology. This being said, there is potential within the pharmaceutical, chemical, cosmetic and hazardous waste industries that may expand *HazardScreen*'s application.

According to a market leader in water testing products (*Danaher Corporation*) the global water testing market is worth approximately US\$2.2B¹.

HazardScreen technology has the potential to be applied to a range of tests that would not only tap into this already large market but we think that the simplicity, portability and cost effectiveness of the technology will expand this market to include previously unreachable consumers.

The need for supplies of fresh water is growing at an alarming rate due to the rapid growth in world population and the proliferation of industry and the resulting effects on the environment.

Currently there are 1.1 billion people worldwide without access to safe drinking water.² These issues are most apparent in developing countries where access to a cost effective and simple method of monitoring water quality would be highly beneficial.

The *Global Environmental Outlook (2000)*³ ranks fresh water availability and water pollution as two of the most important concerns facing developing countries.

The most urgent issues raised in the GEO were:

- In Africa 14 countries are subject to water stress or water scarcity, and a further 11 will join them by 2025;
- One in three people in Asia do not have access to safe drinking water; and
- In West Asia population is increasing faster than water resources. 8 out of 11 countries have consumption levels below chronically low levels (1,000 cubic meters per year per capita).

In a survey conducted for the GEO by the *International Council for Science* (Scientific Committee), freshwater scarcity was the second most important issue (behind climate changes). Freshwater pollution ranked third in the same survey.

Water scarcity raises the importance of testing. When available resources are stretched, maintaining their quality is of paramount importance. Increased testing allows for problems to be dealt with in a more timely manner, minimizing any potential harm.

Current tests used to gauge water toxicity involve either expensive equipment or infrastructure along with technical knowledge, and generally they are not portable enough to be completed in the field.

Examples of current bioassays available for use are:

Luminescent bacteria-based test:

¹ *Danaher Corporation* website (www.danaher.com, accessed: 26/07/01)

² World Development Indicators, *World Bank*, 2001, pg. 145.

³ *Global Environmental Outlook 2000*, *The United Nations Environmental Programme*, 2000.

- A well established, bio-test based on the inhibition of bacterial luminescence by toxic substances.
- Luminescent bacteria are exposed to water samples. The luminescence is measured before and after exposition to calculate the inhibition in percent. An inhibition greater than 20% is considered a toxic effect by the sample.
- The details of the procedure can be found in ISO Norm 11348 (DIN 38412 Teil 34 in Germany).
- The *Microtox* test is the most prominent of such tests. *Microtox* is a bioassay for the screening of water sample toxicity using the marine bacterium *Photobacterium phosphoreum*.
- To conduct the test the *Microtox* desktop testing system is required along with a constant supply of luminescent bacteria.

Whole, small marine life based test:

- This involves exposing marine life to samples for a period of time and seeing how many of them survive.
- This method is very time consuming and maintaining a suitable supply of marine life is very costly.
- Furthermore, it involves sacrificing a vertebrate animal to obtain a result.

Submitochondrial particle test:

- An *in vitro* bioassay for aquatic toxicity detecting toxicants such as chlorophenols that affect mitochondrial functions.
- The test is particularly suitable for investigating chemical toxicity and determining the sites and the molecular mechanisms of their action.
- The authors suggest that SMPs are suitable for use as a prescreening bioassay.

The high cost and level of technical knowledge needed to operate these tests and lack of portability exclude a large portion of potential users.

We would further note that, compared to the *HazardScreen* test, which uses a Eukaryotic protein as its risk identifier, each of these tests is based on a more complex form of life, and therefore introduce a level of ambiguity caused by the presence of organism specific systems, structures and sensitivities.

Some examples of consumers who may benefit from a cheaper, accurate, simpler, more portable bioassay include:

- Regional water testing authorities;
- Home consumers;
- Tank water users;
- Recreational service providers;
- Outdoors enthusiasts;
- International/Domestic aid;
- Military;
- Mining and other companies that potentially pollute/contaminate water;
- Farmers;
- Global support groups (e.g. United Nations, World Health Organization)
- Environmental groups;
- Educational institutions;
- Governments of underprivileged countries; and
- Travelers.

Water testing authorities around the world, particularly those in developed countries have been using a battery of tests including those described above to determine the toxicity of water. Batteries of bioassays for the evaluation of complex environmental samples have been widely recommended as superior to a single bioassay, since it is unlikely that a single bioassay will be responsive to all possible toxicants.

HazardScreen technology may overcome some of the need for multiple tests because it uses the interaction between ubiquitous, naturally occurring macromolecules rather than whole organisms or part thereof. This should eliminate any effects that would be specific to the chosen organism.

Existing players

The major element in most of the currently available toxicity tests is technical capability. Because of this, consultants, or permanent skilled staff generally conducts the tests.

Even if someone was able to get hold of the materials to conduct the whole, small marine life based test or the submitochondrial particle test they would not have the expertise to carry it out.

The most directly substitutable technology for *HazardScreen* is the *Microtox* system manufactured by *AZUR Environmental*.

AZUR scientists published a paper in 1979 entitled "*The Use of Luminescent Bacteria for Determining Toxicity in Aquatic Environments*" which established the use of luminescent bacteria as a method for the testing water samples for toxicity.

Toxicity testing is generally offered as a lab-based test performed by skilled personnel using one of the methods described above. Even though the *Microtox* is the simplest of these tests it is generally unusable by laypersons due to the complexities in the analysis of results.

Other competitors, who have products that could be substituted for *HazardScreen* technology in particular circumstances, are those that sell devices, or services to test the chemical make-up of samples.

There are companies that produce an array of devices that will analyze sample composition. These range from small inexpensive devices to test individual elements of samples such as pH (approx. AU\$10-\$20), all the way through to spectrophotometers (approx. AU\$4,000 and upwards) and atomic absorption spectrometers (approx. AU\$50,000 and upwards) that report on a multitude of elements including heavy metals.

A leader in the manufacture of such devices is *Hach*. They manufacture and distribute analytical instruments used to test the quality of water and other aqueous solutions. *Hach* is a subsidiary of *The Danaher Corporation*.

The *Water Quality Group* of *Danaher* is a world leader in analytical instrumentation for water quality and water treatment products. *Danaher* estimates the worldwide water testing market to be worth approximately US\$2.2B⁴ (AU\$4.4B⁵).

⁴ *Danaher Corporation* website (www.danaher.com, accessed: 26/07/01)

⁵ Translated at: US\$ 0.5045 and ST£ 0.3525 respectively. Rates correct as at 26/07/01 according to published retail sell rates in *The Australian Financial Review*.

HazardScreen is confident that the entry cost, usability and functionality of our technology will allow the development of products that will substantially compete in and extend existing competitors markets.

Table 5 outlines the major players in the water testing market. As mentioned above *AZUR (Microtox)* is the only producer of a toxicity test. Other competitors in the market offer possible substitutable products, which test the composition of water samples.

Table 5 - Competitor water testing related turnover

Company	Turnover (local currency) ⁶	Turnover (AU\$) ⁷
AZUR Environmental	US\$4.5M	8.9M
Hach	US\$102M	202.2M
Strategic Diagnostics	US\$24.8M	49.2M
IDEXX Laboratories	US\$73.5M	145.7M
Viridor Instrumentation	ST£54.9M	155.7M
Palintest	ST£32.7M	92.8M

Below is a brief outline of the businesses contained in Table 5.

AZUR Environmental⁸

In 1979, *AZUR* scientists published a paper titled "*The Use of Luminescent Bacteria for Determining Toxicity in Aquatic Environments*" which established the use of luminescent bacteria as a method for testing water samples for toxicity.

From this discovery *AZUR* produced the *Microtox* and related systems. Compared to existing methods of aqueous toxicity testing *Microtox* was substantially more cost effective and easy-to-use. *Microtox* tests are generally carried out by skilled personnel either within testing authorities or on a consulting basis.

To complete the *Microtox* test you require the desktop unit, computer and appropriate software for interpretation of data and a constant supply of luminescent bacteria.

⁶ All turnover amounts relate water testing markets. All US\$ amount are for the year ended 31/12/01. All ST£ amounts are for the year ended 1/07/01.

⁷ Translated at: US\$ 0.5045 and ST£ 0.3525 respectively. Rates correct as at 26/07/01 according to published retail sell rates in *The Australian Financial Review*.

⁸ All information obtained from *AZUR Environmental* website, except where expressly referenced otherwise (www.AZURenv.com, accessed: 26/07/01)

The *Microtox* system is widely accepted as part of a battery of methods used for toxicity testing around the world.

Hach⁹

Hach is a wholly owned subsidiary of *Danaher Corporation*. *Danaher* has an annual turnover of US\$3,777.8M (AU\$7,488.2M¹⁰).

Hach is part of the *Environmental Division of Danaher*. They manufacture and distribute analytical instruments used to test the quality of water and other aqueous solutions.

Products in the *Hach* range include:

- Spectrophotometers;
- Colorimeters;
- Turbidimeters;
- Particle counters; and
- On-line analyzers.

Strategic Diagnostic Inc (SDI)¹¹

SDI's products include kits for pesticide and industrial contaminants, bacterial contamination, toxic by-products of water chlorination and polymers used for water clarification.

These include kits to measure:

- 40 different pesticides in water;
- The presence of the pathogenic protozoa *Cryptosporidium* and *Giardia*; and
- The concentration of water treatment polymers.

They are also in the process of acquiring *AZUR Environmental* (see above). They believe a general toxicity test such as the *Microtox* system produced by *AZUR* would be complementary to their existing product line.

“Once a toxicity test has been completed, if the result is positive, the individual contaminant (e.g. pesticides, trihalomethanes and TCE) tests will be run to confirm the actual contaminants causing the toxicity”.¹²

IDEXX Laboratories¹³

IDEXX provides diagnostic, detection, and information products to the animal health industry as well as quality assurance products and services to the dairy and water industries. Total turnover for 2000 was US\$367.4M (AU\$ 728.2M¹⁴).

⁹ All information obtained from *Hach* website, except where expressly referenced otherwise (www.hach.com, accessed: 26/07/01)

¹⁰ Translated at: US\$ 0.5045 and ST£ 0.3525 respectively. Rates correct as at 26/07/01 according to published retail sell rates in *The Australian Financial Review*.

¹¹ All information obtained from *Strategic Diagnostic Inc* website, except where expressly referenced otherwise (www.sdix.com, accessed: 26/07/01)

¹² *Strategic Diagnostic Inc* 2000 Annual Report

¹³ All information obtained from *IDEXX Laboratories* website, except where expressly referenced otherwise (www.idexx.com/water, accessed: 26/07/01)

¹⁴ Translated at: US\$ 0.5045 and ST£ 0.3525 respectively. Rates correct as at 26/07/01 according to published retail sell rates in *The Australian Financial Review*.

IDEXX entered the water testing market in 1993 with Colilert, a coliform and *E. coli* water test. Colilert is now used more than all other methods for coliform and *E. coli* testing combined in US, Canadian and Japanese drinking water markets.

They offer tests for key drinking water microbiological analytes including:

- Coliforms and *E. coli*;
- Enterococci;
- Heterotrophic Plate Counts (HPC); and
- *Cryptosporidium* and *Giardia*.

Viridor Instrumentation¹⁵

Viridor is a wholly owned subsidiary of *The Pennon Group*.

The Pennon Group is an UK based PLC which operates and invests primarily in the areas of water and sewerage services, waste management and instrumentation¹⁶. Total turnover for *The Pennon Group* in 2001 was ST£435.1M (AU\$1,234.3M¹⁷).

Viridor has three water quality divisions: *Great Lakes Instruments*, *Ele International*, and *Hydrolab*.

1. Great Lakes Instruments (GLI)

GLI manufactures sensors, analyzers and accessories for liquid quality analysis and process control applications.

GLI products include:

- pH/ORP analyzers and sensors;
- Electrodeless and contacting conductivity;
- Turbidity/Particle counters;
- Dissolved oxygen systems;
- Residual chlorine measurement products;
- Residual ozone monitors;
- Flow measurement and control products; and
- Level sensors.

2. Ele International

Ele produce a multi purpose water testing unit called *Paqualab*. The *Paqualab* tests the following water quality parameters:

- Total Coliforms & *E. Coli*;
- pH, Conductivity, Turbidity; and
- Over 40 chemicals including ammonia, chlorine, nitrite, nitrate.

The *Paqualab* enables the key indicators of water quality as specified in EC and WHO guidelines to be measured rapidly and easily.

3. Hydrolab

¹⁵ All information obtained from *Viridor* website, except where expressly referenced otherwise (www.viridor.com, accessed: 26/07/01)

¹⁶ The Pennon Group website (www.pennon-group.com, accessed: 26/07/01)

¹⁷ Translated at: US\$ 0.5045 and ST£ 0.3525 respectively. Rates correct as at 26/07/01 according to published retail sell rates in *The Australian Financial Review*.

Hydrolab manufactures multi-parameter water quality monitoring instrumentation.

Hydrolab's water quality logger, the *Quantra*, is used to monitor water in a variety of applications such as rivers, lakes, reservoirs, streams, bore holes, industrial discharges, fish farming, oceanography and bathing.

The *Quanta* measures the following parameters:

- Temperature;
- pH;
- Conductivity;
- Dissolved Oxygen;
- Redox (ORP); and
- Depth.

Palintest¹⁸

Palintest is a wholly owned subsidiary of *The Halma Group*.

The Halma Group is an UK based PLC manufacturing group. It develops electronic products, which are used to enhance safety and to reduce hazards. It has over 40 subsidiary companies in the UK, continental Europe, North America, Asia and Australasia¹⁹. Total turnover for *Halma* in 2001 was ST£268.3 mil (AU\$761.1 mil²⁰). *Palintest* produces a core product range of pre-packaged analytical tests for water analysis.

They produce a range of technologies suitable for the following applications:

- Comparator methods for basic field tests;
- Photometer methods for accurate monitoring of critical systems;
- Tubetests - liquid reagents for effluent analysis;
- Tablet/Drop count kits for industrial applications; and
- Chemical sensor technology for the detection of heavy metals.

Palintest is the only European manufacturer with US EPA approval for its methods.

¹⁸ All information obtained from *Palintest* website, except where expressly referenced otherwise (www.palintest.com, accessed: 26/07/01)

¹⁹ *The Halma Group* website (www.halma.com, accessed: 26/07/01)

²⁰ Translated at: US\$ 0.5045 and ST£ 0.3525 respectively. Rates correct as at 26/07/01 according to published retail sell rates in *The Australian Financial Review*.

Technology

HazardScreen has developed a test for the detection of toxicants in water that substantially improves on existing methods functionality and usability.

Generally speaking, the test is based on the observation of the inhibiting effects of pollutants on the interaction between the binding partnership of two molecules. For more detailed explanation of this principal and its application please refer to the *Research* section of this document.

The impetus for developing such a test was a realisation that existing methods were too cumbersome and required significant infrastructure and/or skill to conduct, therefore reducing their effectiveness.

With cheap, accurate, easy-to-use tests available, the monitoring net is expanded to all those with an interest in the environment or their personal water consumption.

The desired properties of such test are that it be:

- Biologically relevant;
- Simple;
- Cheap;
- Reproducible; and,
- Portable.

By measuring total toxicity we are able to take into account the effects of multiple toxicants acting together. The interaction of toxicants can have effects that are unknown and far greater than the sum of their individual effects.

Also, it is important to note that when testing for an element such as mercury, *total* Hg can be distinctly different from *free* Hg. Total Hg can be monitored by atomic absorption spectrometry. However, such an analysis provides no real clues to the bio-toxicity of the Hg. This is because a substantial fraction of Hg may not be available to biological systems. Also, we know there are conditions that sensitize biological systems to metal ion contamination.²¹

It is envisaged that *HazardScreen* technology will be utilised in a first-line-of-defense capacity - detecting potentially polluted water that can be subsequently analysed in a laboratory to distinguish the precise chemical makeup and source of the pollution.

With such a simple, accurate, cheap technology *HazardScreen* allows for more efficient and timely allocation of testing resources.

Patent information

The patent abstract (as contained in US patent application number 09/778259) is as follows:

“The present invention relates generally to an assay for the detection of toxicants. More particularly, the present invention contemplates an assay of toxicants such as those of the type comprising heavy and light metals, heavy

²¹ Pollak, J. (1998), *A short review of the problems posed by Xenobiotics in chemical mixtures and the role of mixed function oxidases*, International Journal of Environmental Health Research, Vol. 8, p157-163.

and light metal ions and organic molecules as well as organo-halides. Such toxicants are frequently present as contaminants in aquatic and terrestrial environments. They may also be resident in living organisms following exposure to the toxicants or where toxicants are administered to higher organisms such as during a treatment protocol. The present invention further provides an assay device for detecting toxicants. The present invention is predicated in part on the sensitivity of binding partner affinity to the toxicants.²²

Patent applications

An international patent is pending covering the technology developed by Professor dos Remedios *et al*, which includes all countries in the Patent Co-operation Treaty (PCT).

Full national phase of patent applications for countries in the PCT commenced in August 2002. Patents in the European Union have now been awarded. Final stages are being completed for the US patent.

To complete the protection of the *HazardScreen* technology, a series of individual patents have been sought in countries that fall outside the PCT. A list of the countries where patents are pending is contained in Table 1.

Table 1 – Pending patents for individual countries outside the PCT

Country	Application No.
United States	09/778259
Argentina	P010100548
Chile	2001-291
Venezuela	258-2001
Taiwan	90102649
Malaysia	PI20010531
Thailand	63447
Pakistan	106/2001
The Philippines	I-2001-00246

Full searches have been completed by our patent attorneys. We are confident our core technology is unique.

²² Patent Abstract, *Biological Toxicity Assay*, HazardScreen, 2000.

Research

To date research has focused on two types of test for water pollution, one based on protein-protein interactions; the second involving the use of molecules that fluoresce when they bind to DNA.

Protein based assay

Kekic and dos Remedios have described the use of native-polyacrylamide gel electrophoresis (native-PAGE) for detecting heavy metal and herbicide contamination of water (attached).²³

This technique separates proteins and their complexes on the basis of charge and shape and is non-denaturing. The actin:cofilin complex was monitored in response to a variety of pollutants. The complex runs as a single band on native-PAGE gels which, can also separate actin and cofilin bands when they are not complexed.

The results indicate that the formation of the complex is sensitive to pollutants as determined by the disappearance of the actin:cofilin complex band. These results are detailed in the attached publication.

From the research described above, three conclusions were made:

1. Not all heavy metal ions have identical effects on actin-cofilin dissociation, some (e.g. Hg) being more toxic than others (e.g. Cd);
2. The electrophoretic test was sensitive to Hg, Cu, Cd and Zn ions in nearly the same order and with comparable sensitivity to other conventional bioassays for water pollution (these data are summarised in Table 2); and
3. The test was also sensitive to other pollutants such as herbicides (e.g. picloram).

Table 2 - EC₅₀ (50% toxicity) concentrations for Hg, Cu, Cd and Zn²⁴

Metal Cation	Actin-Cofilin $\mu\text{g L}^{-1}$	SMP $\mu\text{g L}^{-1}$	BHM $\mu\text{g L}^{-1}$	Fish $\mu\text{g L}^{-1}$ (at 96 hr)	Microtox $\mu\text{g L}^{-1}$
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

*SMP, BHM, Fish and Microtox data described by Read et al, in Microscale Testing in Aquatic Toxicology -Advances, Techniques and Practice.*²⁵

²³ Kekic M, dos Remedios CG (1999) Electrophoretic monitoring of pollutants: effect of cations and organic compounds on protein interactions monitored by native gel electrophoresis. *Electrophoresis* **20**:2053-8.

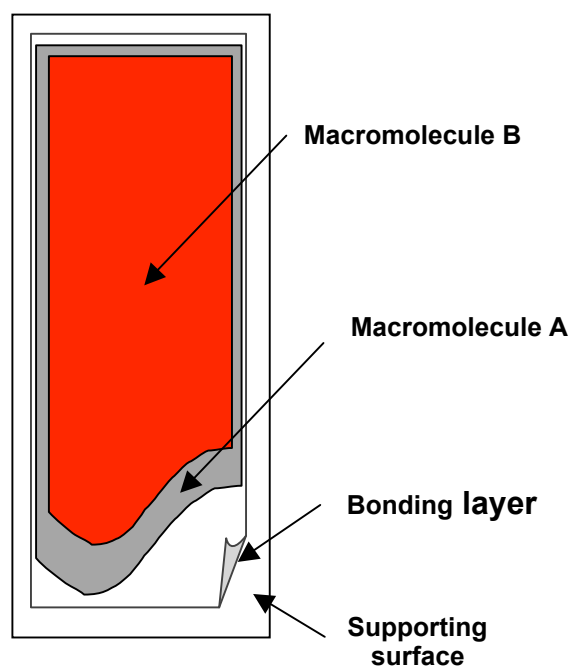
²⁴ Determined by: inhibition of actin-cofilin binding as observed in native gel PAGE; inhibition of submitochondrial particle assay; inhibition of a beef heart mitochondrial activity; a whole fish toxicity assay; and the *Microtox* test based on inhibition of luminescence of a strain of bacteria.

²⁵ Read HW, Harkin JM, Gustavson KE. 1997. In: *Microscale Testing in Aquatic Toxicology -Advances , Techniques and Practice*. Wells PG, Lee K, Blaise C (eds). CRC Press, Boca Raton.

Strip test

This technique was extrapolated to a matrix-based (dry strip) assay. The dry strip test was intended to develop in the form of a plastic or nitrocellulose strip on which one protein, (e.g. cofilin) was either covalently linked or otherwise permanently immobilised. The second protein (e.g. actin) is pre-labelled with a dye that is readily visible and added to the first protein and the complex is air or freeze-dried. The concept of this test is shown in Figure 1 below.

Figure 1 - Experimental design for a dry strip assay using cofilin and labelled actin bound to a nitrocellulose membrane.



Recombinant cofilin was immobilized on a solid bead-type support (glutathione agarose) and a fluorescently labeled (Texas Red™ dye) actin was passed through the beads. A proportion of the actin bound to the cofilin and was immobilized on the beads giving them a distinct colour. Passing a non-toxic solution through the matrix did not remove the dye.

A solution of 100µm mercury removed the dye from the matrix. The mercury disrupted the interaction between actin and cofilin and thus the dyed actin was eluted, however there was insufficient dye intensity (the color of the bound actin was very pale pink) for it to be readily visible by eye.

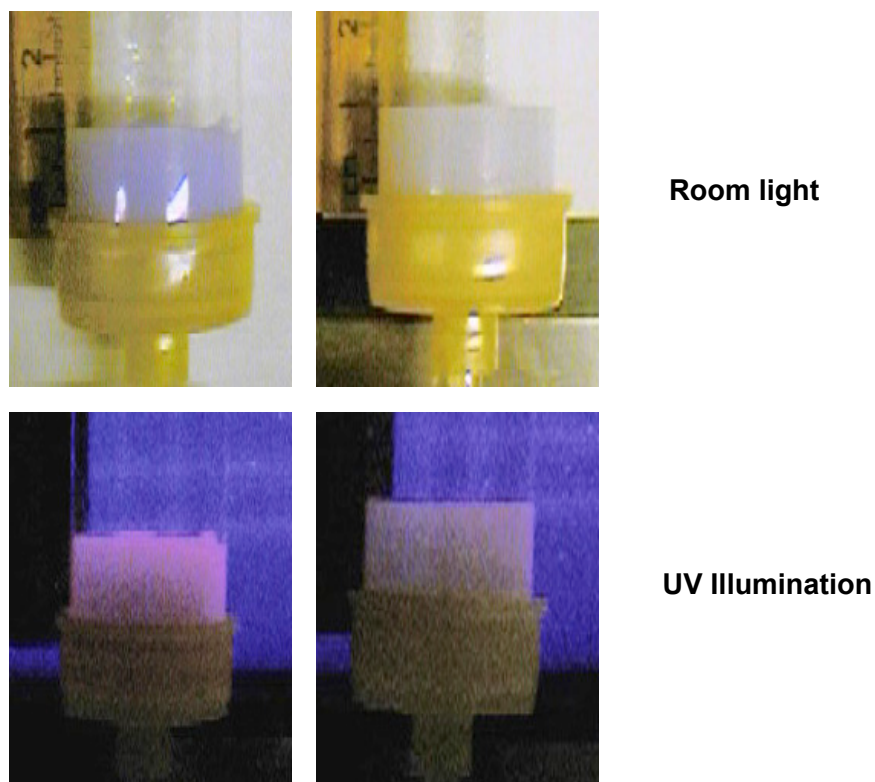
Development of this form of the test is going to require substantial experience in the plastic and membrane industry and is probably embodied in the form of 'trade secrets'.

Column test

A second form of the protein-bases pollution assay was developed using immobilised cofilin on a column. This is a relatively straightforward process since this protein is a recombinant one made in the bacterium, *E. coli*, and is synthesised with the GST tag needed for column immobilisation.

Actin was labelled with the dye Texas Red and then added to the column. This is actually a blue colour when observed by eye but it fluoresces intensely in the red frequencies when illuminated by near-UV light. The physical arrangement of the dyes in short chromatography columns are illustrated below in Figure 2.

Figure 2 - Chromatographic assay that uses immobilised cofilin (not colored) and Texas Red-labelled actin (appears blue).



Top two figures show the assay viewed in room light. The lower two figures illustrate the fluorescence in the absence (left) and presence (right of Hg). Note the loss of fluorescence (pink) in the column shown on the bottom right.

DNA based assay

In an extension of the protein based assay described above, we developed a bioassay based on the ability of mercury (Hg²⁺) to quench fluorescence of ethidium bromide (EB) bound to DNA (refer attached).

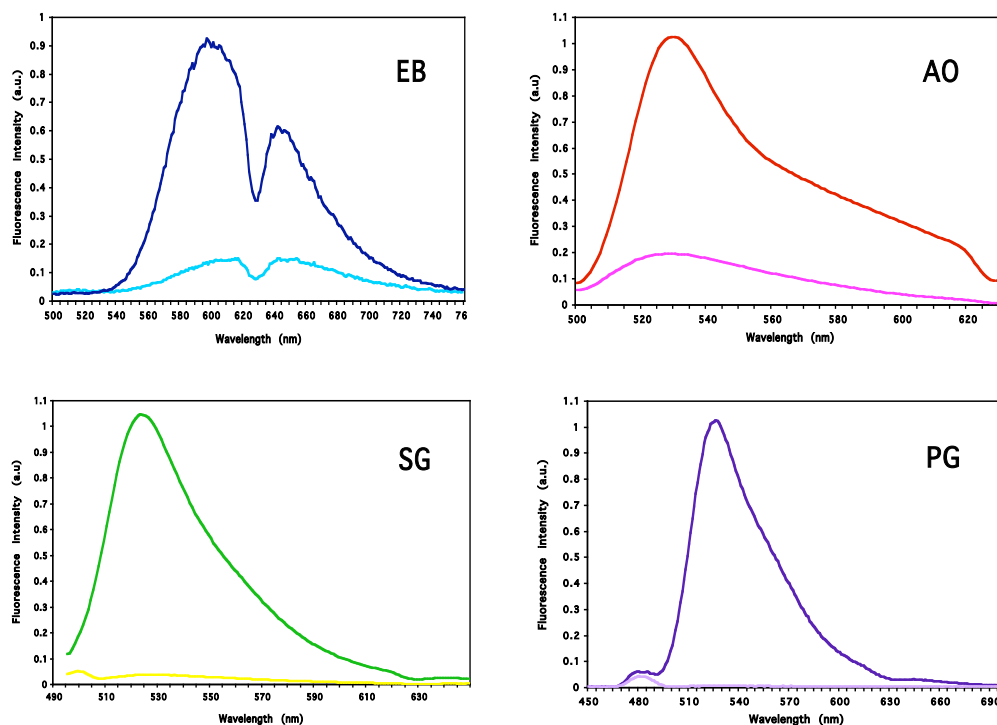
DNA is a macromolecule with enormous potential as a bioassay. It has several properties that make it desirable, including:

- DNA is present in all living organisms and is a ubiquitous indicator of environmental pollution;
- Damage to DNA structure has a detrimental effect to the existence of any organism;
- DNA structure is changed by a variety of agents;
- DNA is stable for extremely long periods in a dry state; and
- DNA is relatively inexpensive.

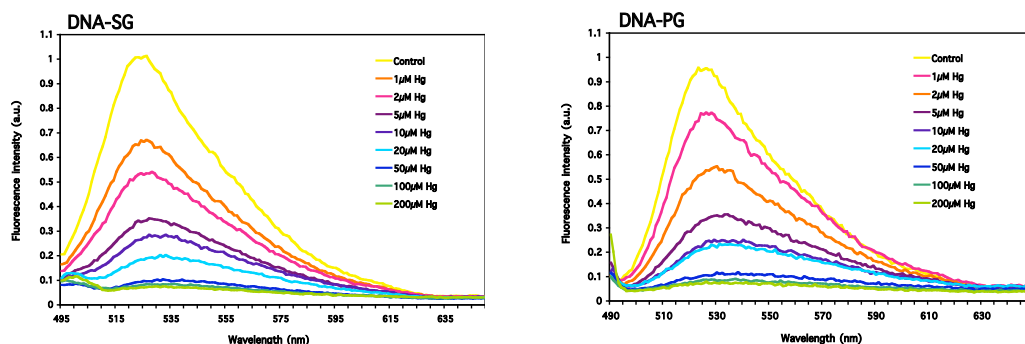
DNA can bind a variety of fluorescent dyes (i.e. ethidium bromide - EB, acridine orange - AO, SYBR green I – SG, PicoGreen - PG) but only when in its native

double-stranded state. These dyes exhibit preferential and exceptional affinity for double stranded DNA and produce a large fluorescence enhancement when they bind (see Figure 3).

Figure 3 - Fluorescence excitation and emission spectra for complexes of DNA with ethidium bromide (EB), acridine orange (AO), SYBR Green I (SG) and PicoGreen (PG)



EB, when excited at 475 nm, emits a weak fluorescence with a peak around 600 nm. However when EB binds to DNA the fluorescence emission intensity increases over 6 fold. AO exhibits a similar increase in fluorescence to EB when bound to DNA. The fluorescence of SG and PG is almost undetectable at the emission peak at 520 nm, but increases by almost 30 fold when it binds to DNA. Each dye has distinctive fluorescence excitation and emission spectra exhibiting different excitation and emission maxima.



The basis of this bioassay is that the structure of DNA is altered by a variety of pollutants. The ability of a fluorescent dye to bind to DNA is governed by the structure of the DNA. When toxins are added to the DNA-dye complex, the DNA undergoes a structural change causing the dye to be released.

Therefore, the fluorescence of the DNA-dye complex is quenched when exposed to a toxic substance.

The Effects of Mercury on the DNA-Dye Complex

Mercury is released into water primarily by chemical and mining industries. Long-term exposure causes damage to kidneys and other organs.

Figure 4 - Effects of Hg on the fluorescence emission of four DNA-binding dyes

As can be seen in figure 4, the fluorescence emission intensity of DNA-EB is measurably decreased in the presence of only 2 μ M Hg. A similar quenching effect was observed for DNA-AO, DNA-SG and DNA-PG fluorescence emission, however DNA-AO exhibited a biphasic response, where the initial decrease in fluorescence was followed by an increase at higher concentrations of Hg.

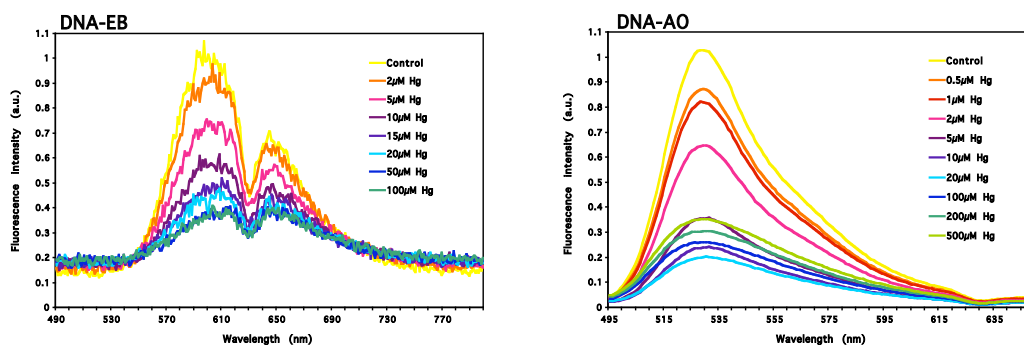
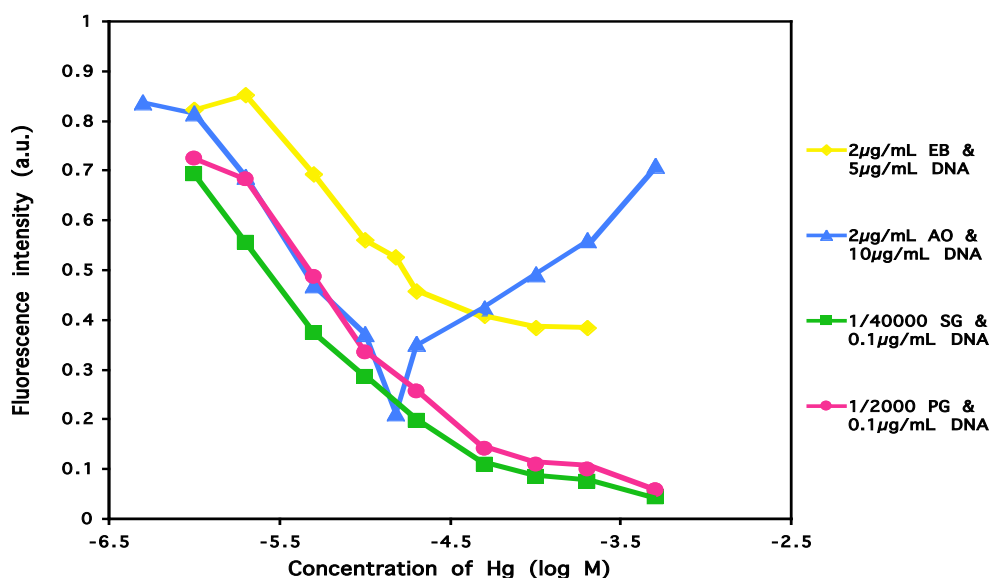


Figure 5 - Concentration dependent effects of Hg on the fluorescence emission of four DNA-binding dyes



As can be seen from Figure 5, DNA-SG and DNA-PG are the most sensitive to Hg, DNA-AO has a biphasic response and DNA-EB is the least sensitive of the four dyes. From this, the EC₅₀ concentration for Hg was determined for each DNA-dye pair.

Table 3 - A comparison of the EC₅₀ concentrations of DNA-dye complexes with the “forward” and “reverse” reactions of the submitochondrial particle assay for Hg ions.

Test type	DNA-EB	DNA-AO	DNA-SG	DNA-PG	SMP Forward reaction ²⁶	SMP Reverse reaction ²⁷
EC ₅₀ (µM)	5.1	2.3	2.3	2.2	38.9	0.6

Table 3 shows that the DNA-PG assay is more sensitive by a factor of about 2 compared to DNA-EB and is only slightly more sensitive than DNA-AO and DNA-SG. The standard error values for the measurements (n = number of determinations) shows that AO, SG and PG are significantly lower than EB.

The SMP forward reaction tests evaluates the ability of a pollutant to uncouple a “chain” of enzymes linked to a segment of mitochondrial membrane. The potential toxic effect was evaluated by determining their inhibitory effects on the oxidative functions of SMP prepared from beef heart mitochondria. This assay is widely accepted as a test for toxicity.

The DNA-PG assay is 17 times more sensitive than the SMP forward reaction test however, the SMP reverse reaction assay is significantly more sensitive. Both these tests are lab based and require significant expertise and both must be performed in a laboratory setting.

The Effects of Heavy Metals on the DNA-PG Complex

The sensitivity of the DNA-PG fluorescence to mercury suggested it might also be sensitive to other heavy metals. Figure 6 shows the effect of increasing concentrations of a variety of heavy metals on the fluorescence of the DNA-PG complex.

The results clearly show that DNA is more sensitive to mercury than other heavy metals. The DNA-PG test is decreasingly sensitivity to other metals in the following order:

$$\text{Hg} > \text{Cr} > \text{Zn} > \text{Cu} > \text{Pb} > \text{Cd} > \text{Ba}$$

Heating the DNA in the presence of cadmium for 20 min at 40°C, assisted in elucidating the toxic effects of Cd. Heating the DNA in the presence of the other heavy metals has no effect on the DNA assay (data not shown) since DNA structure is quite stable at this temperature.

The results obtained using the DNA-dye assay approximately reflect the order toxicity of heavy metals observed by other toxicity tests, such as SMP and *Microtox*. Although there are differences, all tests show that mercury is the most toxic heavy

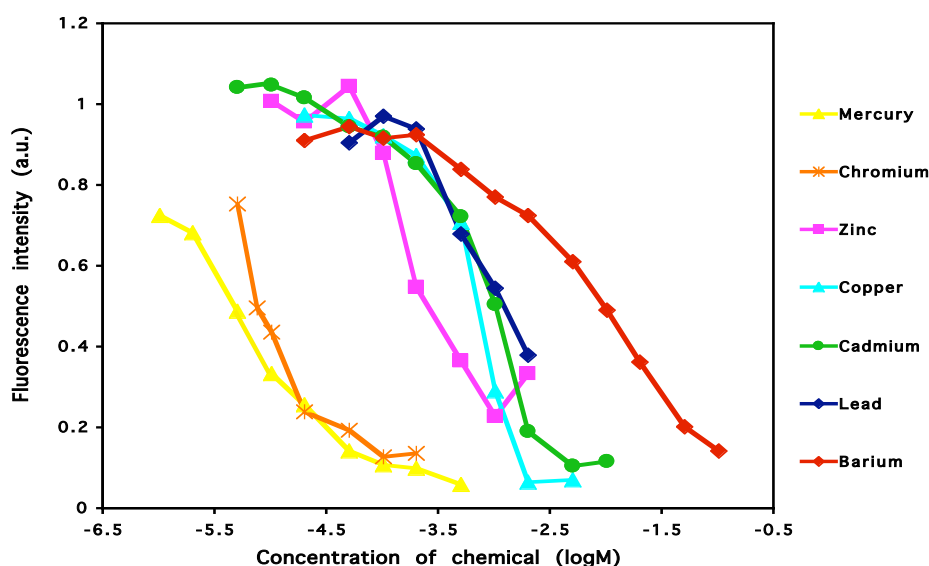
²⁶ Results from tests carried out at The Institute for Biomedical Research, The University of Sydney by Dr Diana Oakes and Dr John Pollak using the same samples used to conduct DNA-dye tests.

²⁷ Read HW, Harkin JM, Gustavson KE. 1997. In: *Microscale Testing in Aquatic Toxicology -Advances , Techniques and Practice*. Wells PG, Lee K, Blaise C (eds). CRC Press, Boca Raton.

metal ion and Ba is least toxic. The order of heavy metal toxicity according to the SMP and *Microtox* tests is as follows: ²⁸

SMP Hg > Cd > Cu > Pb > Zn > Cr > Ba
 Microtox Hg > Pb > Cu > Cr > Cd > Zn > Ba

Figure 6 - Concentration-response relationship between the concentration of the above heavy metals and the fluorescence intensities of DNA-PG in solution.



The above responses were measured at the emission peak with the excitation wavelength set at 480 nm. From these curves the EC₅₀ can be determined.

Table 4 - EC₅₀ concentrations for the heavy metals in Figure 6

Heavy metal	Hg	Cr	Zn	Cu	Pb	Cd	Ba
EC ₅₀ (µM)	2.2	9.1	174	673	745	860	4403

The results indicate the DNA-dye complex is highly sensitive to the presence of heavy metal cations, thus providing an indicator of pollution.

Membrane-Based DNA-EB Assay

DNA binds very strongly to certain types of membranes. We were able to develop a solid-state assay by drying DNA-EB onto strips of these membranes and detecting the fluorescence of EB.

Incubating the strips with mercury caused a quenching of the fluorescence within 30-60 minutes. The quenching continued to gradually decline until it reached equilibrium at 24 hours.

The sensitivity, compared to the solution assay, was also reduced by a factor of 6 (EC₅₀ 32 µM). These long assay periods required for the development of this assay greatly complicate the utility of this test.

As with the development of solid state protein assay the techniques and technologies involved are of a 'trade secret' nature. With access to these capabilities, it is envisaged that the DNA-dye assay could be applied to a solid support thus increasing its in-the-field functionality.

Funding

The cost of research was initially funded in 1999 by a \$180,000 donation from Strategic Ventures. This company also provided in kind services for the development of a business plan and for accounting services of approximately \$50,000.

In 2000, a grant of \$120,000 was awarded by the Hermon Slade Foundation (Australia). This project was entitled: "A Molecular Device for the Detection of Aqueous Environmental Pollution". These funds provided research assistance and research costs for the project over a period of three years ending in 2003.

Patent costs of over \$160,000 were provided by Strategic Ventures and a further \$40,000 was contributed to these costs by Mr Gary Leech. Total: \$200,000

A partial scholarship (\$40,000) for a PhD student working on the project was provided by C.G. dos Remedios.

NSW State and Regional Development has provided matching funding of up to \$68,000 for the development of a marketing plan.

The NSW Environmental Protection Agency (EPA) has provided in kind assistance in the form of technical assistance, collection of polluted water samples, costs of analyses (\$125,000).

The total funding provided to this project to the end of 2003 exceeds \$780,000.

Hazard Screen Pty Ltd is currently seeking a commercial partner to contribute \$500,000 to the future development of this project.