Australian Government Analytical Laboratories (AGAL)

Drugs in Sport Research Plan 2001-2005

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AGAL DRUGS IN SPORT RESEARCH PLAN 2001-2005

1. Objective

AGAL's Drugs-in-Sport Research Program is designed to maintain and expand the level of expertise developed by the Australian Sports Testing Laboratory (ASDTL) in the lead up to the Sydney 2000 Olympic Games, and further promote the fight against doping in sport.

2. Background

The Australian Sports Drug Testing Laboratory (ASDTL) was established in 1988 to provide a comprehensive sports drug testing capability in Australia and the surrounding region. ASDTL is the only IOC accredited laboratory in Australasia, and one of only 25 worldwide. As one of the agencies involved in the Government's *Tough on Drugs in Sport* strategy, ASDTL continues to be at the forefront of sports drug detection through the use of state-of-the-art technology and an innovative research and development program.

In 2000 ASDTL carried out the drug testing for the Sydney 2000 Olympic and Paralympic Games and in so doing introduced two new drug-testing methodologies, one for detecting doping with recombinant erythropoietin (EPO) and one for detecting doping with endogenous hormones such as testosterone. These methods were developed with funds provided by the Sydney 2000 Olympic Games Drug Research Program. This research program commenced in 1997 with the aims of developing improvements in analytical techniques that will increase our ability to detect banned substances and also investigating new forms of doping that were currently undetectable. Apart from the short term benefit of introducing new tests at an Olympics there have been lasting benefits to Australia's and the world's fight against drugs in sport. These benefits include:

- The establishment of high resolution mass spectrometry for the detection of low level steroids as part of ASDTL's routine methodology. ASDTL was the first IOC laboratory to obtain ISO accreditation for this technique.
- Presentation of data from the statistical profiling project relating to "normal" levels of nandrolone metabolites supporting the correctness of the IOC 2ng/mL limit.
- The introduction of carbon isotope ratio mass spectrometry as part of ASDTL's routine methodology (also ISO accredited) for detecting and confirming the abuse of endogenous anabolic steroids. Application of this method at the 2000 Paralympic Games enabled the banning of two athletes for testosterone abuse. This was the first time that such bans have been imposed at the time of a major event because of the previous need for extensive follow up studies to confirm testosterone doping.
- The blood and urine test for recombinant EPO is now being routinely used by ASDA for targeted testing.
- The development of assays for growth hormone isoforms has supported the development of a robust test for growth hormone.

As a result, the profile of Australia's fight against doping in sport has been considerably enhanced. The provision of funding by the International Olympic Committee (IOC) for

the EPO2000 project and subsequent grants from the World Anti-Doping Agency (WADA) are evidence of the confidence in Australia's anti-doping research strategy.

3. Research Plan 2001-2005

Reliability and currency of sports drug-testing procedures must be maintained if Australia's anti-drugs in sport program is to remain effective. AGAL's research program is aimed both at developing improvements in analytical techniques that will increase our ability to detect banned substances and also at investigating new forms of doping which are currently undetectable.

The World Anti-Doping Agency has defined three categories under which applications for research funding were divided. The categories are:

A. Factors regulating and enhancing growth.
B. Compounds enhancing the oxygen carrying capacity of the blood.
C. Endogenous testosterone, testosterone precursors and metabolites, 19 nor-steroids and establishment of normal urinary levels of these and related compounds.

The primary focus of the AGAL research plan is to complement and expand WADA's aims by making use of the research skills available within ASDTL and its Australian collaborators. The main goals of the plan are to:

- Complete and extend projects begun prior to the Sydney Olympics;
- Complement and accelerate the progress of projects funded by WADA;
- Prepare for new instrumental demands; and
- Reserve some funds for new developments over the four-year period of the plan, including support for further applications for external funding from WADA and the IOC.

The current AGAL drugs-in-sport research program is both budget-funded, through AGAL's National Interest Program, and funded by external sources such as the IOC and WADA.

In addition to projects already funded, funds are requested through *Backing Australia's Sporting Ability (BASA)* to enable some of the partially funded projects above to proceed to completion and to fund new projects.

The projects in this research plan are listed in Table 1. These research projects were developed following a review of the scientific literature and developments currently underway in ASDTL and other IOC laboratories, taking particular note of the WADA's long term goals. The aim has been to capitalise on the pool of expertise and equipment developed in the lead up to the Sydney 2000 Olympic Games. Whilst there were significant breakthroughs made during this period there is now the need to continue the

work so as to maintain the momentum and uphold the high reputation that Australia has achieved in the fight against doping in sport.

One of the major areas to be developed is the automation and simplification of newly developed tests such as the combined blood/urine test for recombinant EPO (Sydney protocol). Unless there are significant improvements it will not be possible to apply such tests as widely as desired because of their high cost and complexity.

Additional projects for years 2, 3 and 4 will be developed depending on emerging testing requirements.

Table 1: AGAL Drugs-in-Sport Research Projects

Research Projects	WADA Research Category [1]	AGAL Project No.	WADA Project No.	Source of Funding
Robust Test for Growth Hormone (Collaboration)	A	P2002-01	A3	WADA & BASA
Mass Spectrometry of Peptide Hormones	A and B	P2002-02		BASA
Recombinant EPO in Urine [2]	B	P2002-03		BASA
Extension of Statistical Profiling	B	P2002-04		BASA
Detection of the Abuse of Hb based Blood Substitutes in Sport	B	P2002-05	B1	WADA
Urinary EPO Interlaboratory trial	B	P2002-06		IOC
Analysis of Sports Supplements	C	P2002-07		BASA
Statistical Profiling	C	P2002-08		AGAL
CIRMS Interlaboratory Study	C	P2002-09		BASA
CIRMS Profiling Study	C	P2002-10		BASA
Carbon Isotope Ratio Ring Tests (Collaboration)	C	P2002-11	C3	WADA
Preparation of Certified Reference Materials for Drugs in Sport Analysis	C	P2002-12	C8	WADA
LC/MS Applications	D	P2002-13		AGAL
IOC/WADA Reaccreditation	D	P2002-14		AGAL

[1] WADA Research Categories:
- A: Factors regulating and enhancing growth
- B: Compounds enhancing the oxygen carrying capacity of the blood.
- C: Endogenous testosterone, testosterone precursors and metabolites, 19 nor-steroids and establishment of normal urinary levels of these and related compounds.
- D: Other

[2] In conjunction with the Australian company Proteome Systems, a world leader in the design and manufacture of gels. Proteome Systems have recently obtained a Biotechnology Innovation Fund grant to develop gels specifically for the detection of recombinant EPO.

4. Project Summaries – Backing Australia's Sporting Ability

P2002-01 Robust Test for Growth Hormone – Defining Interactions Between Anabolic and Peptide Hormones

This collaborative project, with the principal investigator located at the Garvan Institute of Medical Research, is a three year program and approximately two thirds of the funding required has been provided by WADA ($400,000 USD for Year 1; $400,000 USD for Years 2 and 3 dependent on progress).

The major thrust of this proposal is to develop a robust test(s) for growth hormone (GH) doping in sport. The specific aims are to:

1. Investigate interactions between GH, EPO and androgenic steroids which may be relevant to implementation of doping tests.
2. Continue the evaluation of molecular isoforms of GH (22k, 20k, and 17k) as a direct test for GH doping.
3. Define the reference range of potentially useful markers of GH in elite athletes utilising an existing databank of more than 4000 samples collected from elite athletes of diverse ethnic origins.

However, WADA funding for this project is insufficient to undertake all the work elements proposed. In addition, the project collaborators believe that the prioritisation of work components requested by WADA will result in long delays between different facets of the research. Additional funding through BASA will allow complete, well integrated planning of the project, greatly enhancing the chance of a test for GH being available for the 2004 Olympic Games.

BASA Funding Requested:

	Project Costs
Year 1	$238,000
Year 2	$238,000
Year 3	$238,000

P2002-02 Mass Spectrometry of Peptide Hormones

The aim of this project is to establish and develop a facility which is capable of meeting the increasing demands for confirmation of peptide hormones and other large biologically active molecules using liquid chromatography mass spectrometry (LC/MS). This facility will be required to underpin the development and cost effective application of new tests for both growth hormone, EPO and blood substitutes.

Without developments in the use of mass spectrometry, laboratories will have to continue to make measurements using immunoassays, which can always be challenged on the grounds of cross reactivity. The IOC rules mass spectrometry as the definitive method for confirmation except in the case of peptide hormones. However, it is now possible to do so for peptide hormones but requires specialised equipment and techniques that are only just becoming commercially available.

It has been seen with the development of new techniques such as carbon isotope ratio mass spectrometry that, once a mass spectral technique can be shown to replace or supplement a less rigorous procedure, its use becomes essential both to confirm positive results. The same will apply to the peptide hormones in the near future and positive cases will require mass spectral confirmation once it is possible to do so.

The equipment required for this work is in part available within ASDTL, but new developments will require additional equipment in years 2 and 3 of the project.

BASA Funding Requested:

	Project Costs	**Capital Equipment**
Year 1	$256,000	$20,000
Year 2	$350,000	$400,000
Year 3	$400,000	$400,000
Year 4	$428,000	$50,000

Capital equipment is for a minor upgrade to of a Finnigan MAT900 high resolution mass spectrometer in year 1, with a replacement instrument required in year 2 or 3, at an estimated cost of $800,000.

P2002-03 Recombinant EPO in urine

This proposal aims to simplify the methodology used in the current EPO urine test (the French isoelectrophoresis test as used at Sydney 2000 Olympic Games as part of the EPO 'Sydney protocol' test) so that it can be applied to more samples at substantially lower cost.

Before any significant improvements can be made a more reliable source of gels is needed. The French urine test for EPO, which uses a gel electrophoresis method for the detection of recombinant EPO, uses gels that are prepared within the laboratory. Such gels lack robustness and the results obtained on one gel will not necessarily be exactly the same as those from another gel even prepared in the same laboratory. The inter-laboratory trial of the French urine EPO method funded by the IOC has confirmed the problems that are encountered with the laboratory-prepared gels. The first stage of this process will be to develop a robust immobilised pH gradient (IPG) gel in collaboration with an Australian company, Proteome Systems, a world leader in this technology.* The gel will be used in IOC laboratories to improve the reproducibility of results, particularly between laboratories, obtained using the gel electrophoresis test for recombinant EPO.

If no improvements are made the existing method for the detection of recombinant EPO in urine will remain a confirmation method unsuitable for routine screening. Successful completion of the steps outlined above should simplify and automate the methodology so that it can be economically applied to high volume screening.

It is then proposed to expand the method so that it includes the new EPO replacement NESP (Novel Erythrocyte Stimulating Peptide). At the same time work will begin on selectively extracting EPO from both blood and urine with a view to simplifying the existing complex double blotting process and enabling the method to be applied to blood. It is also proposed to use the mass spectrometry techniques developed in the project above to attempt the detection and confirmation of recombinant EPO, NESP and other EPO mimetics.

BASA Funding Requested:

	Project Costs
Year 1	$142,000

* Proteome Systems is a world leader in this technology. They have recently obtained a Biotechnology Innovation Fund grant to develop a gel or gels specially designed for the detection of recombinant EPO.

P2002-04 Extension of Statistical Profiling

Several of the substances banned by the IOC are produced as endogenous compounds in the human body. This requires parameters to be established whereby it is possible to distinguish between administered drug and natural production. This requires measurements from many samples collected over a period in order to determine population statistics and monitor normal distributions amongst various population groups. With the addition of endogenous compounds such as EPO to the range of compounds that can be detected it is now necessary to have more information relating to the natural levels found in urine and blood where available. With time this will also enable the monitoring of individual athletes over time.

The French method to detect recombinant EPO in urine, whilst definitive, relies on the difference in glycosylation and hence basicity between recombinant and normal urinary EPO. At present the number of samples from elite athletes that have been tested to establish the normal levels is quite small (a few hundred). Thus there is a need to expand this and improve the statistical reliability of the test and perhaps lower the limit at which doping can be confirmed. The more data that is available to confirm that the basicity observed from recombinant EPO administration is outside the range of that found in a large population of elite athletes, the sooner the urine test will stand alone as proof of EPO doping. This will significantly reduce the cost of EPO detection and enable the blood test to be simplified and optimised for selecting those samples on which to run the EPO urine test. There is also no long-term data on the variation of EPO levels and EPO basicity in urine over time for individuals.

BASA Funding Requested:

	Project Costs
Year 1	$122,000

P2002-07 Analysis of Sports Supplements

The increase in positive drug samples due to the presence of metabolites of nandrolone is largely due to the presence of compounds such as norandrostenedione or norandrostenediol in commercially available dietary supplements. The compounds norandrostenedione and norandrostenediol, whilst banned in sport by the IOC, are freely available as health food products in the USA and there have been several cases of dietary supplements being contaminated with these compounds. If such a supplement is taken it is likely that the athlete consuming it will test positive to the metabolites of nandrolone, as the metabolites of norandrostenedione and norandrostenediol are the same as those of nandrolone.

There is no current means by which Australian sporting bodies or athletes can learn which dietary supplements pose a risk. Ethical problems prevent AGAL providing this service on a fee-for-service basis. Such results, if negative, could be seen to be a case of AGAL endorsing a particular product or group of products. This study proposes confidential reporting of the results to ASDA, with the results of this survey published without providing manufacturers information. This is similar to a program now being carried out by the Cologne IOC laboratory for supplements available in Europe.

This process will inform athletes about the level of contamination of supplements available on the Australian market and reinforce any trends found overseas. Confidential reports including details of supplement names and manufacturers could be provided to ASDA and the ASC to assist in the prevention of inadvertent doping.

BASA Funding Requested:

	Project Costs
Year 1	$117,000

P2002-09 Carbon Isotope Ratio Mass Spectrometry Inter-laboratory Study

This is an ongoing project that commenced in January 2001 with the ultimate goal of achieving agreement among IOC-accredited doping laboratories on a carbon isotope ratio (CIR) method for the detection of administered endogenous substances, and with the IOC/WADA on the use of CIR.

All IOC accredited laboratories are required not only to analyse routine samples but also to contribute to the development of sports drug testing by carrying out research into the detection of new substances. Since ASDTL was the first IOC laboratory to use CIR/MS methodology at a summer Olympic Games and the first to use the technique to confirm testosterone doping at a major event (the Paralympics) there is an opportunity to remain at the forefront of the use of CIR/MS. There is an obvious need for inter-laboratory studies to be conducted amongst those IOC laboratories with CIR/MS in order to ensure comparability of results between laboratories. AGAL is well placed to undertake this assignment, as ASDTL will provide analytical input and the National Analytical Reference Laboratory (NARL) the expertise on reference standard preparation.

Stage 1 of the study will result in an initial comparison of accuracies and uncertainties in results from those laboratories employing CIR methods for detecting endogenous steroid abuse, and provide a starting point for discussion on method improvement.

BASA Funding Requested:

	Project Costs
Year 1	$151,000

P2000-010 Carbon Isotope Ratio Mass Spectrometry Profiling Study

This is an ongoing project that commenced in January 2000 as part of the Olympic Research program with the aim of measuring the isotope ratio of the natural steroids found in the urines of over 1000 elite athletes. Its purpose is to produce additional data to support the use of CIRMS in detecting doping with endogenous steroids. This data will be crucial in setting decision limits for the detection of doping using CIRMS.

Detection of endogenous steroid doping using carbon isotope mass spectrometry is a reasonably recent addition to the range of tests used in the IOC accredited laboratories. Despite having been used at the Nagano Winter Games (1998) and the Sydney 2000 Summer Games, the test is still somewhat experimental, with most of the laboratories that employ the technique working independently of each other to develop required criteria. Because the test relies on detecting differences in the isotope ratio of steroids present in the body it is necessary to know the normal values found in a large population of elite athletes living in different countries. The reason for this is that isotope ratio is known to vary with diet and subjects of different ethnic origin may have adopted the diet of the country in which they reside.

Approximately 100 samples have been analysed. The available funding finished on June 30 2001 and failure to continue will mean the project will terminate with most of the samples remaining unanalysed. An opportunity will be lost to produce a statistically significant set of results from a range of elite athletes around the world. The lack of such published data will make the routine implementation of CIRMS much more difficult to achieve.

BASA Funding Requested:

	Project Costs
Year 1	$140,000

5. Project Summaries – WADA-, AGAL- and IOC-Funded Projects

P2002-05 Detection of the Abuse of Hb based blood substitutes (WADA)

The aim of this project is to develop a simple method or methods to detect the presence of haemoglobin-based substitutes in blood with high selectivity and sensitivity, and to develop a confirmatory method that can withstand legal challenge. Haemoglobin-based blood substitutes have the ability to carry oxygen to the tissues whilst providing rapid expansion of blood volume. They are being developed as a replacement for blood transfusions in emergencies and their properties make them eminently suitable for performance enhancement in endurance sports. Only one product is currently approved for human use and it is hoped to have methods in place in time to act as a deterrent and hence minimise their misuse in sport. The test will not work with urine and will require blood collection.

Funding: $140,000 USD ($A 270,000) over 1 to 1.5 years.

P2002-06 Urinary EPO Interlaboratory trial (IOC)

The aim of this project was to carry out a full validation of the French method for detecting recombinant EPO in urine. This was necessary to fully characterise the method by determining the set of parameters affecting the results and hence assure the legal defensibility of the method. The protocol consisted of two parts:

1. Evaluation of previous data and intra-laboratory validation
2. Inter-laboratory comparison.

Five IOC laboratories took part in the study Paris, Barcelona, Sydney, Oslo, and Lausanne. Blind spiked urine samples for use in the study were prepared by NARL. A report on the results has been prepared and submitted to the IOC.

Funding: $100,000 USD between the five laboratories over 1 year. AGAL received $A 60,000 for this project.

P2002-08 Statistical Profiling (AGAL)

This is an ongoing project that aims to complete the development of, and maintain a database of some endogenous substance levels in routine athlete samples. This will enable constant monitoring of the normal distributions amongst various population groups. The data obtained will be published and will provide previously unavailable data as to how parameters such as T/E ratios vary over time in a large group of elite athletes. The same data will also enable monitoring of individual athletes over time.

Funding: $76,000 for one year.

P2002-11 Carbon Isotope Ratio Ring Tests (WADA)

After seven years of basic research, carbon isotope ratio mass spectrometry has advanced from an experimental technique to a practical test for detecting the use of testosterone and other steroids. This project focuses on improving carbon isotope ratio measurements and showing that, whilst the procedure details may differ from one laboratory to another, the final result will be the same. The aim is to improve the ability of IOC-accredited laboratories to measure delta values by conducting three rounds of ring tests among five IOC laboratories. These laboratories are Beijing, Penang, London, Los Angeles, and Sydney. The protocol will be designed to systematically investigate variables deemed to be important in the analysis and will make much use of calibration standards to ensure that both accuracy and precision are maintained.

Funding: This project has been granted $510,700 USD between the five laboratories over three years. $170,000 USD for year 1 is approved with funding for years 2 and 3 dependent on progress. AGAL will receive $A 60,000 of this funding.

P2002-12 Preparation of Certified Reference Materials for Drugs in Sport (WADA)

The aim of this project is to produce Certified Reference Materials (CRMs) of marker metabolites for testosterone, testosterone precursors and19-norsteroids. Laboratories undertaking testing and research in this area need these materials. Their availability will contribute to improving the quality assurance procedures, the intercomparability and the assessment of measurement uncertainty in such analyses, and to fundamental research into the qualitative and quantitative detection of abuse of such compounds. Ten CRMs will be produced for doping tests involving androstenedione, DHEA, testosterone, or 19-norsteroids.

Funding: $510,000 over 1.5 years. $380,000 for year 1 is approved, with $130,000 for year 2 dependent on progress.

P2002-13 LC/MS Applications (AGAL)

The primary aim of this project is to introduce liquid chromatography mass spectrometry (LC/MS) into routine sports drug testing. The first goal will be to convert the existing GC/MS screen for diuretics to an LC/MS method. The new method should detect a wider range of diuretics including new diuretics that are difficult to detect with the existing method. It also has the potential to simplify the existing extraction and derivatisation procedure. It is anticipated that the new method will result in higher quality at lower cost.

Funding: $125,000 for one year.

P2002-14 IOC/WADA Reaccreditation (AGAL)

This is an ongoing project that provides for the work needed to prepare for the IOC/WADA reaccreditation process each year. The IOC accreditation is the fundamental requirement to enable ASDTL to undertake testing of athletes for doping agents. Each year new drugs are added to the list of banned substances and each year the IOC require laboratories to undertake a proficiency test which may include these new substances. The Doping Subcommission and a panel of external experts then review the results.

The results from this work need to be published and presented at the most appropriate forum, which is the Cologne Workshop held each year. This way new methods and results are rapidly transferred to other IOC laboratories for implementation. This is important to ensure worldwide coverage of the anti-doping program. This project aims to enable procedures for analysis of new substances to be developed and to maintain ASDTL's IOC accreditation status.

Funding: $99,000 for one year.

Appendix 1 Summary of AGAL Drugs-in-Sport Research Plan 2001-2005

Project No.	Research Projects	WADA Category[1]	AGAL[2] $'000	BASA[2] $'000					WADA[2] $'000					IOC $'000	Project Total $'000
			Yr 1	Yr 1	Yr 2	Yr 3	Yr 4	**Total**	Yr 1	Yr 2	Yr 3	Yr 4	**Total**	Yr 1	
P2002-01	Robust Test for Growth Hormone (Collaboration)[3]	A		238	238	238		**714**	800	800*	800*		**2400**		**3,114**
P2002-02	Mass Spectrometry of Peptide Hormones	A, B		276	750	800	478	**2304**							**2304**
P2002-03	Recombinant EPO in Urine	B		142				**142**							**142**
P2002-04	Extension of Statistical Profiling	B		122				**122**							**122**
P2002-05	Detection of the Abuse of Hb-based Blood Substitutes	B							270				**270**		**270**
P2002-06	Urinary EPO Interlaboratory trial	B												60	**60**
P2002-07	Analysis of Sports Supplements	C		117				**117**							**117**
P2002-08	Statistical Profiling	C	76												**76**
P2002-09	CIRMS Interlaboratory Study	C		151				**151**							**151**
P2002-10	CIRMS Profiling Study	C		140				**140**							**140**
P2002-11	Carbon Isotope Ratio Ring Tests (Collaboration)[2]	C							60	60*	60*		**180**		**180**
P2002-12	Preparation of CRMs for Drugs in Sport Analysis	C							380	130*			**510**		**510**
P2002-13	LC/MS Applications	D	125												**125**
P2002-14	IOC/WADA Reaccreditation	D	99												**99**
	TOTAL		**300**	**1186**	**988**	**1038**	**478**	**3398**	**710+**	**190+***	**60+***		**960***	**60**	

Notes:

[1] WADA Research Categories:
- A: Factors regulating and enhancing growth
- B: Compounds enhancing the oxygen carrying capacity of the blood.
- C: Endogenous testosterone, testosterone precursors and metabolites, 19 nor-steroids and establishment of normal urinary levels of these and related compounds.
- D: Other

[2] Additional projects for years 2, 3, and 4 will be developed depending on needs arising in the previous year.

[3] Funds shown are those expected to be available to AGAL, except for P2002-01 where the amounts allocated to each of the five partners will vary in each year of the project, with AGAL's share likely to be less than 20% on occasions.

* Funds should be available but depends on the results achieved in Year 1.

Appendix 2 Detailed Project Proposals – Backing Australia's Sporting Ability

P2002-01 Robust Test for Growth Hormone

Summary of Project Purpose and Aims:

The major thrust of this proposal is to develop a robust test(s) for growth hormone (GH) doping in sport. The specific aims are to:

1. Investigate interactions between GH, EPO and androgenic steroids, which may be relevant to implementation of doping tests.
2. Continue the evaluation of molecular isoforms of GH (22k, 20k, and 17k) as a direct test for GH doping.
3. Define the reference range of potentially useful markers of GH in elite athletes utilising an existing databank of more than 3000 samples collected from elite athletes of diverse ethnic origins.

This is a three-year program and approximately two thirds of the funding required has been provided by WADA. Additional funding is sought in order to complete the program.

Planned Outcome(s):

A reliable test for GH doping will enable IOC laboratories to test for growth hormone and hence there will be less use of GH by athletes.

Proposed Output(s):

1. A report to WADA and a peer reviewed publications on the:
    - effect of EPO administration on proposed GH markers;
    - range of GH marker values (including isoform values) found in over 3000 samples collected from elite athletes around the world; and
    - results obtained from an administration trial of combined GH and testosterone to approximately ninety subjects.

2. A reliable test for GH doping based on the results obtained from the above three studies.

Project Background

The proposed studies are partially based on the initially promising work that came from the GH2000 project. The WADA has recognised that our consortium is capable of developing a robust test based on our expertise and resources.

Following the work of GH2000 two approaches were proposed for detecting GH doping, one using indirect markers of GH action and one based on the quantitation of GH isoforms. There is a now a need to validate the application of these techniques and develop a robust test for the detection of GH doping. The difficulties of the development of tests for doping are compounded by the fact that athletes may take performance-enhancing drugs from more than one class. This project proposes to examine how physiological and pharmacological interactions will impact on the predictive value of a drug test for GH. For example anabolic steroids stimulate the GH system, and both anabolic steroids and GH stimulate erythropoiesis. Defining these interactions will help establish a robust test for GH doping. There are four parts to the project:

1. EPO effects on GH/IGF-1 axis (existing samples)
2. Defining normal parameters from databank (existing samples)
3. 17k GH ELISA development
4. GH-Testosterone study

The first two parts of the project will determine whether the indirect markers proposed by GH2000 can be used in an effective manner to detect GH doping. The development of an assay for the 17k GH isoform will complement the existing 20k assay developed by the group and help determine whether a combination of isoform specific assays can be used in the direct detection of GH doping. The last part of the project involving a large administration study will help answer questions about the detection of GH doping particularly when GH is taken in conjunction with anabolic steroids.

Technical Background:

The development of a test for GH abuse has led to two approaches one indirect and one direct. The indirect approach relies on the measurement of relatively long lasting markers of GH such as IGF-1. However in order to apply such an indirect test the normal range of these markers must be established by measuring levels in a large population of elite athletes from different countries. ASDTL has stored samples from just such a population of elite athletes collected during the EPO2000 study. Approval has been obtained from the subjects for such analyses to be conducted.

The direct approach to the detection of GH doping relies on the fact that GH is secreted as several isoforms of differing molecular weight including 5k, 17k, 20k and 22k. Recombinant GH has a single isoform namely 22k. Thus the detection of unusual isoform distributions could detect and confirm GH doping. To do this one needs both specific assays for the isoforms and access to a databank of samples from elite athletes to establish normal isoform distributions. Our group has both of these available now and is intending a to refine an existing 17k assay to improve the robustness of the method.

The administration study will make use of all the information gained form the earlier parts of the study and will help provide answers to questions relating to the effectiveness of GH doping alone and in conjunction with anabolic steroids such as testosterone. It will provide data on how GH doping could impact on the detection of EPO doping.

Project Plan – Year 1

#	*Objective*	*Target*
1	Key positions appointed	November 2001
2	EPO administration studies sorted	February 2002
3	Database established	February 2002
4	20 and 22k GH assayed	May 2002
5	GH markers assayed	May 2002
6	Statistical analysis completed	July 2002
7	Databank samples sent to appropriate laboratories	June 2002
8	Monoclonal antibody screen completed	June 2002
9	Report to WADA on EPO administration study samples	October 2002
10	Recruitment started for GH/testosterone administration study	September 2002
11	17k ELISA assay established	October 2002

Project Expenses

Year 1 [1]

Project Expenses	
Staffing and Administration	$107,000
Consumables	$77,000
Depreciation, Cost of Capital, Repairs and Maintenance	$800
Travel [2]	$6,000
Other [3]	$7,200
Facilities	$35,000
Quality Assurance Framework	$5,000
TOTAL	**$238,000**

Notes:

[1] Project expenses are expected to be similar in years 2 and 3.

[2] Meetings with overseas collaborators.

[3] Includes the use of an external statistician to examine data.

P2002-02 Mass Spectrometry of Peptide Hormones

Summary of Project Purpose and Aims:

To establish a facility within ASDTL which is capable of meeting the increasing demands for confirmation of peptide hormones and other large biologically active molecules using liquid chromatography mass spectrometry (LC/MS).

Planned Outcome(s):

An ongoing capability to develop and implement mass spectral methods for the detection and confirmation of doping with peptide hormones and other large bio-molecules.

Proposed Output(s):

1. A validated method to confirm cases of HCG doping using LC/MS.
2. A LC/MS method to identify and confirm the presence of haemoglobin based blood substitutes.
3. A mass spectral method to distinguish between recombinant EPO and urinary EPO (links with Project P2000/03)
4. Mass spectral methods to detect and identify other significant biologically active molecules such as NESP, EPO mimetics, growth hormone isomers, IGF1 etc (links with Project P2000/01, P2000/03 and P2000/05).

Project Background

There is an urgent need to develop the skill and resource base needed to carry out the mass spectral analysis of bio-molecules used for doping. Whilst at present the use of immunoassays and other immuno-reactive techniques is accepted as proof of doping this is unlikely to continue once it has been demonstrated that mass spectral confirmation is possible. Recently published work has shown that it is now possible to detect and identify proteins in biological matrices at the extremely low concentrations found naturally. It has been seen with the development of new techniques such as carbon isotope ratio mass spectrometry that, once a mass spectral technique can be shown to replace or supplement a less rigorous procedure, its use becomes essential both to confirm guilt and to demonstrate innocence. The same will apply to the peptide hormones in the near future and positive cases will require mass spectral confirmation once it is possible to do so.

ASDTL purchased a high resolution mass spectrometer capable of analysing bio-molecules with funds from the Olympic research program. However, now this program has now finished and there are insufficient staff and resources to continue the research work needed to develop the skills required for the analysis of large molecules by mass spectrometry. It has become apparent that mass spectral analysis will not only be needed for the analysis of peptide hormones but also for identifying and confirming the

presence of other biologically active compounds such as the haemoglobin based blood substitutes.

Because of the Olympic research program ASDTL is well placed to continue and expand on its work on the detection of large bio-molecules such as EPO. The immediate need is for staff to continue this work by expanding our skill base in the field of large molecule mass spectrometry. The current equipment we have is capable of demonstrating proof of concept and developing new methods but will need to be replaced or supplemented in 2002 or 2003 because of the rapid developments in mass spectrometric instrumentation. By then it is likely that the instruments will be at least an order of magnitude more sensitive and selective. Research instruments already exist which theoretically could for example distinguish recombinant from urinary EPO but their cost is prohibitive. However, within the time frame of 2001 to 2004 this is likely to change with routine commercial instruments becoming available with similar or superior capabilities.

Technical Background:

At present the confirmation of the presence of peptide hormones and other large bioactive molecules is done using techniques that rely on specific antibody reactions to large molecules. Unfortunately, such reactions are not completely specific and the current IOC/WADA anti-doping code includes the need for two separate antibodies to confirm doping with HCG. All drugs that are detected, other than peptide hormones, must be confirmed by the use of mass spectrometry using gas chromatography mass spectrometry. The reason the peptide hormones were excluded from this requirement was that it was not practicable to attempt mass spectrometric analysis of large bio-molecules both because of their high molecular weight and because of the very low concentrations found in blood and urine. However with the ever increasing demands of proteomics research the use and capabilities of mass spectrometry using LC/MS for the analysis of bio-molecules has increased dramatically in the last few years and will continue to do so. Already one IOC laboratory has presented a method, using LC/MS, which is capable of quantitating and confirming the presence of HCG at the very low levels found in urine.

Project Plan

#	*Objective*	*Target*
1	Staff member recruited to establish LC/MS facility	November 2001
2	Method developed and validated for the detection of HCG in urine	February 2002
3	Validated method for the confirmation of a range of blood substitutes	July 2002
4	Paper prepared on possibility of distinguishing recombinant from urinary EPO by MS	June 2003
5	Method developed for detection of IGF1	June 2003
6	Purchase of new LCMS	July 2003
7	Methods developed for GH isoforms in blood	June 2005
8	Methods developed for EPO mimetics	June 2004

Project Expenses

Year 1

Project Expenses		Capital Equipment	
Staffing and Administration	$172,000	Minor upgrade to High Resolution Mass Spectrometer	$20,000
Consumables	$12,000		
Depreciation, Cost of Capital, Repairs and Maintenance	$17,000		
Travel	$6,500		
Other	$5,500		
Facilities	$38,000		
Quality Assurance Framework	$5,000		
TOTAL	**$256,000**		**$20,000**

Year 2

Project Expenses		Capital Equipment	
Staffing and Administration	$240,000	New High Resolution Mass Spectrometer (1st Instalment)	$400,000
Consumables	$25,000		
Depreciation, Cost of Capital, Repairs and Maintenance	$15,000		
Travel	$8,000		
Other	$5,000		
Facilities	$52,000		
Quality Assurance Framework	$5,000		
TOTAL	**$350,000**	**TOTAL**	**$400,000**

Year 3

Project Expenses		Capital Equipment	
Staffing and Administration	$257,000	New High Resolution Mass Spectrometer (2nd Instalment)	$400,000
Consumables	$42,000		
Depreciation, Cost of Capital, Repairs and Maintenance	$12,000		
Travel	$16,000		
Other	$8,000		
Facilities	$60,000		
Quality Assurance Framework	$5,000		
TOTAL	**$400,000**	**TOTAL**	**$400,000**

Year 4

Project Expenses		Capital Equipment	
Staffing and Administration	$275,000	Instrument upgrades	$50,000
Consumables	$42,000		
Depreciation, Cost of Capital, Repairs and Maintenance	$9,000		
Travel	$8,000		
Other	$25,000		
Facilities	$64,000		
Quality Assurance Framework	$5,000		
TOTAL	**$428,000**		**$50,000**

P2002-03 Recombinant EPO in Urine

Summary of Project Purpose and Aims:

To simplify the methodology used so that the French EPO urine test can be applied to more samples at substantially lower cost. This will take place in a number of stages:

1. In collaboration with Proteome Systems develop a robust immobilised pH gradient (IPG) gel that can be used in all IOC laboratories to improve reproducibility of results, particularly between laboratories, obtained using the gel electrophoresis test for recombinant EPO developed by the French IOC laboratory;
2. Expand the method so that it includes the new EPO replacement NESP (Novel Erythrocyte Stimulating Peptide);
3. Investigate means of selectively extracting recombinant EPO from both urine and blood. This should have the dual benefit of removing the need for the complicated double blotting process and enabling the test to be applied to blood; and
4. Investigate the use of mass spectrometry to detect and confirm the presence of recombinant EPO. This links with Project P2002-02.

Planned Outcome(s):

A simpler and cheaper test for EPO doping will result in better detection by more IOC laboratories and less use of EPO by athletes.

Proposed Output(s):

1. A commercially available IPG gel that can be used in the test for recombinant EPO and NESP; and
2. Closer correlation between the results obtained in difference IOC laboratories using the EPO urine test.

Project Background

The test for recombinant EPO in urine developed by our colleagues at LNDD in Paris, whilst able to detect the abuse of EPO, has a number of significant limitations. They all relate to the complexity of the method, which means that:

- the production of results is slow and cannot always be guaranteed
- the reproducibility between laboratories is not as high as desired
- the cost of the method is so high that it is impractical to test as many samples as required.

A more robust and simpler method is needed. If no improvements are made to the existing method for the detection of recombinant EPO in urine then it will remain a confirmation method unsuitable for routine screening. The year one program is the first stage in a series of steps to simplify and automate the methodology so that it can be economically applied to high volume screening. The first step in this long process is to obtain a reliable source of electrophoresis gels used in the first stage of the method.

Technical Background:

The gel electrophoresis method for the detection of recombinant EPO in urine developed by LNDD in Paris uses gels which are prepared by the laboratory. Such gels lack robustness and the results obtained on one gel will not necessarily be exactly the same as those from another gel even prepared in the same laboratory. The inter-laboratory trial of the French urine EPO method, funded by the IOC, has confirmed the problems that are encountered with the laboratory prepared gels. The newer immobilised pH gradient gels are now used for most electrophoretic separation of proteins. AGAL does not have the technical expertise to produce such gels but a nearby Australian company, Proteome Systems, is a world leader in this technology. They have recently obtained a Biotechnology Innovation Fund grant to develop a gel or gels specially designed for the detection of recombinant EPO. ASDTL will provide the knowledge and skills needed to test the gels provided and incorporate them into the urine test for recombinant EPO.

The availability of a reliable commercial gel will significantly improve the robustness of the method but will only marginally reduce the overall cost and complexity of the method. The double blotting process used in the method is a major source of the methods complexity and cost. Alternative means of separating the recombinant EPO from other proteins will be examined. These will include the use of immunoaffinity columns and immobilised antibodies. If this approach is successful it will not only simplify the method but also mean that it could be applied to blood as well as to urine. It is known that serum EPO concentrations correlate much more closely with EPO use than do urinary EPO concentrations.

Another stream of the research will look at using modern instrumental techniques such as liquid chromatography mass spectrometry to supplant or complement the immunoaffinity techniques currently used to detect and characterise the presence of EPO. Whilst such equipment is expensive it has the potential to remove many of the steps in the current method and hence reduce its cost and improve its robustness.

Project Plan

#	*Objective*	*Target*
1	First IPG gel tested	October 2001
2	Correct pH range gel chosen	December 2001
3	Validation of EPO method with IPG gel	April 2002
4	Extension of method to include NESP	June 2002
5	Paper presented at Cologne workshop	March 2002
6	Commercial gels made routinely available to other IOC laboratories	July 2002
7	Paper submitted for publication	August 2002

Project Expenses

Year 1

Project Expenses	
Staffing and Administration	$90,000
Consumables	$15,000
Depreciation, Cost of Capital, Repairs and Maintenance	$700
Travel	$6,000
Other	$6,800
Facilities	$21,000
Quality Assurance Framework	$2,500
TOTAL	**$142,000**

P2002-04 Extension of Statistical Profiling

Summary of Project Purpose and Aims:

To extend the database of endogenous substances to enable constant monitoring of the normal distributions amongst various population groups. With the addition of endogenous compounds such as EPO to the range of compounds that can be detected it is now necessary to have more information relating to the natural levels found in urine and blood, where available.

Planned Outcome(s):

An improvement in the detection of doping with endogenous compounds and a resultant reduction in their abuse by athletes.

Proposed Output(s):

1. An Access database established and updated on a regular basis, containing endogenous compound data for selected ASDA samples over the next one to two years;
2. A paper to be published in a peer-reviewed journal giving a summary of the findings related to the variability of parameters in a large group of elite athletes over a period of one to two years; and
3. Data which may assist in the effective selection of those samples on which to run the full urine test.

Project Background

The method to detect recombinant EPO in urine, whilst definitive, relies on the difference in glycosylation and hence basicity between recombinant and normal urinary EPO. At present the number of samples from elite athletes that have been tested to establish the normal levels is quite small (a few hundred). Thus there is a need to expand the available data and improve the statistical reliability of the urine test and perhaps lower the limit at which doping can be confirmed. The more data that is available to confirm that the basicity observed from recombinant EPO administration is outside the range of that found in a large population of elite athletes, the sooner the urine test will stand alone as proof of EPO doping. This will significantly reduce the cost of EPO detection and enable the blood test to be simplified and optimised for selecting those samples on which to run the EPO urine test. There is also no long-term data on the variation of EPO levels and EPO basicity in urine over time for individuals.

Technical Background:

Several of the substances banned by the IOC are produced as endogenous compounds in the human body. This requires parameters to be established whereby it is possible to distinguish between administered drug and natural production. This requires measurements from many samples collected over a period in order to determine population statistics. Also, where multiple samples can be linked to an individual it is possible to use the tighter individual variation as a means of monitoring drug abuse. Whilst the electrophoretic method for the detection of recombinant EPO in urine does produce a pattern which is not found in normal urinary EPO there is still a need to establish the range of EPO basicities that can occur in normal urines. The values and their range determine the point at which doping with recombinant EPO can be proven. As more data is collected the statistics should improve, meaning that the sensitivity of the test can be increased thus extending its retrospectivity. Repeat samples on individuals will determine whether there is significant variability of EPO basicity for an individual over time.

Project Plan

#	*Objective*	*Target*
1	Measurement of EPO levels in routine ASDA samples started	September 2001
2	Commencement of routine running of one gel per week to establish EPO basicity variation	October 2001
3	Athlete data entered into database	August 2002
4	Preliminary report for Cologne workshop	March 2002
5	Paper prepared for publication	August 2002
6	Completion of 2000 EPO in urine measurements	August 2002

Project Expenses

Year 1

Project Expenses	
Staffing and Administration	$60,000
Consumables	$28,000
Depreciation, Cost of Capital, Repairs and Maintenance	$4,000
Travel	$6,000
Other	$500
Facilities	$21,000
Quality Assurance Framework	$2,500
TOTAL	**$122,000**

P2002-07 Analysis of Sports Supplements

Summary of Project Purpose and Aims:

To determine the extent to which dietary supplements freely available in Australia are likely to cause positive drug tests in athletes. This will be a survey and is not intended to provide guarantees for any product on the market but to provide a general overview of the current situation as it stands in Australia. Various products (with input from ASDA, AIS and a general random selection) will be purchased and checked for contamination by IOC banned steroids. The results of the analytical work will be published as a general survey of products obtained in Australia.

Planned Outcome(s):

Fewer cases of inadvertent doping due to better knowledge of contamination levels in sports supplements available in Australia.

Proposed Output(s):

1. A confidential report to ASDA and ASC summarising the findings so that athletes can be advised of the risks associated with particular supplements; and
2. A peer-reviewed paper published on the results obtained with statistics on the type and frequency of contaminants found.

Project Background

The increase in positive drug samples due to the presence of metabolites on nandrolone is largely due to the presence of compounds such as norandrostenedione or norandrostenediol in commercially available dietary supplements. The compounds norandrostenedione and norandrostenediol, whilst banned in sport by the IOC, are freely available as health food products in the USA and there have been several cases of dietary supplements being contaminated with these compounds. If such a supplement is taken it is likely that the athlete consuming it will test positive to the metabolites of nandrolone as the metabolites of norandrostenedione and norandrostenediol are the same as those of nandrolone.

Technical Background:

There are no current means by which Australian sporting bodies or athletes can learn which dietary supplements pose a risk. Athletes, sporting bodies, and manufacturers have all called for the testing of dietary supplements to determine if they are contaminated.

This study proposes confidential reporting to ASDA, which is similar to a program now being carried out by the Cologne IOC laboratory for supplements available in Europe. This process will inform athletes about the level of contamination of supplements available on the Australian market and reinforce any trends found overseas. The work

performed on this project will develop a routine method for testing supplements, which could be continued to provide ongoing confidential information to ASDA and the ASC.

Project Plan

#	*Objective*	*Target*
1	Method developed and validated for the detection of contaminants in dietary supplements	November 2001
2	Samples purchased for survey	November 2001
3	Analysis completed for 30 supplements	February 2002
4	Preliminary report to ASDA and ASC	February 2002
5	Preliminary results presented at Cologne Workshop	March 2002
6	Further 100 analyses performed and paper presented for publication	August 2002

Project Expenses

Year 1

Project Expenses	
Staffing and Administration	$69,000
Consumables	$12,000
Depreciation, Cost of Capital, Repairs and Maintenance	$4,500
Travel	$6,000
Other	$5,500
Facilities	$17,500
Quality Assurance Framework	$2,500
TOTAL	**$117,000**

P2002-09 Carbon Isotope Ratio (CIR) Mass Spectrometry Inter-laboratory Study

Summary of Project Purpose and Aims:

This is an ongoing project started in January 2001 with the ultimate goal of achieving agreement among IOC-accredited laboratories on a CIR method for the detection of administered endogenous substances, and with IOC/WADA on the use of CIR.

Planned Outcome(s):

1. Agreement between laboratories on approaches to the method eg. use of internal standards, requirements for calculation of acetate contribution (if used), quality control measures and some standardised cutoffs and criteria for analysis;
2. Agreement among laboratories on protocols for use of screening and confirmation CIR methods; and
3. Agreement with the IOC and WADA concerning the protocol for use of CIR and steroid screen results.

Proposed Output(s):

Stage 1: A report on the results sent to all participating laboratories. The report will include:

1. Proficiency of laboratories in measuring the isotope ratios of known hydrocarbon standards;
2. Proficiency of laboratories in measuring ketosteroids and steroid diols;
3. Variety and prevalence of standards used by the laboratories;
4. The range of criteria used for selecting samples for isotope analysis; and
5. The range of criteria used in deciding if a sample is positive.

Stage 2: A report on the results sent to participating laboratories. The report will include:

1. the isotope ratios of proposed reference materials for the steroid metabolites of interest;
2. the isotope ratios of proposed reference materials for the standards;
3. an indication of the variation in isotope measurements between laboratories; and
4. the value of the use of internal standards.

Project Background

The project has already commenced with funding provided by the Olympic Research program. The first set of samples was distributed at the Cologne Anti-doping workshop in March 2001 and fourteen laboratories have tentatively agreed to participate. The available funding finished on June 30 2001 and failure to continue will mean the project will terminate with its first stage incomplete. Stage one of the study will result in an

initial comparison of accuracies and uncertainties in results from those laboratories employing carbon isotope methods for detecting endogenous steroid abuse, and give a starting point for discussion on method improvement.

Technical Background:

Detection of endogenous steroid doping using carbon isotope mass spectrometry is a recent addition to the range of tests used in the IOC accredited laboratories. Despite having been used at the Nagano Winter Games (1998) and the Sydney 2000 Summer Games, the test is still somewhat experimental, with most of the laboratories that employ the technique working independently of each other to develop required criteria. In order to avoid the possibility of conflicting results issued from different laboratories, a more cohesive approach to carbon isotope testing is required.

It is envisaged that the study will consist of an introduction and three phases, each in two parts, those being analysis and discussion, to which participants are expected to contribute. The discussion will take place at the Cologne meeting each year and will consist of two sections, the first discussion will aim to elicit agreement concerning protocols in those samples analysed in the preceding year and the second will relate to analyses to be carried out in the following year.

Project Plan

#	*Objective*	*Target*
1	Analysis of Stage 1 samples completed	August 2001
2	All Stage 1 results received from participating laboratories	September 2001
3	Interim report on data sent to participating laboratories	December 2001
4	Final results of Stage 1 presented and discussed at Cologne anti-doping workshop	March 2002
5	Design of Stage 2 of study based on results and discussions with Stage 1 participants	May 2002
6	Preparation of standard materials for Stage 2 started	June 2002

Project Expenses

Year 1

Project Expenses	
Staffing and Administration	$90,000
Consumables	$8,500
Depreciation, Cost of Capital, Repairs and Maintenance	$14,500
Travel	$6,000
Other	$7,000
Facilities	$22,500
Quality Assurance Framework	$2,500
TOTAL	**$151,000**

P2002-10 Carbon Isotope Ratio (CIR) Mass Spectrometry Profiling Study

Summary of Project Purpose and Aims:

This is an ongoing project started in January 2000 with the aim of measuring the isotope ratio of the natural steroids found in the urines of over 1000 elite athletes collected during the EPO2000 study. Its purpose is to produce additional data to support the use of CIR/MS in detecting doping with endogenous steroids.

Planned Outcome(s):

More data on the natural variation of CIR/MS values from a wide range of athletes will help set legally defensible criteria for the application of the technique.

Proposed Output(s):

A paper in a peer-reviewed journal giving the range and variability of isotope ratios found in approximately 1000 samples collected from elite athletes in 13 countries.

Project Background

The use of CIRMS to detect doping with compounds that occur naturally in the body depends on the ability to distinguish synthetic compounds from naturally occurring ones by the slight differences in the ratio of carbon 12 to carbon 13. This ratio is not fixed but varies slightly depending mainly on the type of food that is consumed. Urine samples collected during the EPO2000 project are from a range of ethnic groups and are available to be analysed but current staff and resources are not adequate to do this promptly. The project has already begun with funding provided by the Olympic Research program. Approximately 100 samples have been analysed, however the available funding finished on June 30 2000.

Technical Background:

Detection of endogenous steroid doping using carbon isotope mass spectrometry is a reasonably recent addition to the range of tests used in the IOC accredited laboratories. Despite having been used at the Nagano Winter Games (1998) and the Sydney 2000 Summer Games, the test is still somewhat experimental, with most of the laboratories that employ the technique working independently of each other to develop required criteria. Because the test relies on detecting differences in the isotope ratio of steroids present in the body it is necessary to know the normal values found in a large population of elite athletes living in different countries. The reason for this is that isotope ratio is known to vary with diet and subjects of different ethnic origin may have adopted the diet of the country in which they reside.

Project Plan

#	*Objective*	*Target*
1	Analysis of 300 samples completed	December 2001
2	Analysis of 600 samples completed	March 2002
3	Analysis of 900 samples completed	June 2002
4	Preliminary results presented at Cologne anti-doping workshop	March 2002
5	Results evaluation completed and paper prepared for publication in peer-reviewed journal	August 2002

Project Expenses

Year 1

Project Expenses	
Staffing and Administration	$88,000
Consumables	$8,500
Depreciation, Cost of Capital, Repairs and Maintenance	$14,000
Travel	$6,000
Other	$1,000
Facilities	$21,000
Quality Assurance Framework	$2,500
TOTAL	**$141,000**