# GLOBAL PROGRESS TOWARD TRANSGENIC FOOD ANIMALS: A SURVEY OF PUBLICLY AVAILABLE INFORMATION

**By**

**Gregory S. Harper***
**Alan Brownlee**
**Thomas E. Hall**
**Robert Seymour**
**Russell Lyons**
**Patrick Ledwith**

CSIRO Livestock Industries, 306 Carmody Rd., St. Lucia, Qld 4067

*To whom correspondence should be addressed. Email address: Gregory.harper@csiro.au

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## Executive Summary

The last decade of animal research has reinforced the view that some food-related qualities of animal products (e.g. eating quality and yield) are strongly influenced by the genotype of the animal. Likewise animal production performance characteristics (e.g. growth rate and disease resistance) have been improved by selective breeding. One significant advantage of transgenesis is in rapid genetic improvement of traits of interest, and a dramatic example of its impact can be seen in transgenic coho salmon that grow up to 11 times faster than their non-transgenic conspecifics. Nonetheless there are legitimate science-based concerns about the impact of these new methods on the production systems and the environments in which they grow as well as the food supply chains.

In this review, we describe the field of animal transgenesis with special reference to food animals. We have collected information by thorough searches of the scientific literature, the popular press and Internet websites. This information was supplemented with personal interviews of thirty-seven practicing research scientists from a range of research and teaching organisations. We now describe transgenic modifications of five terrestrial production species, and several transgenic mouse lines that are informative for developments in these five species. We also describe transgenic developments in thirteen aquaculture species. In each case we have made extensive use of published information, and have cited these references for the reader. The use of transgenic animals for xenotransplantation or human bioactive protein production is considered only briefly. A real issue for consideration by the regulatory authorities is whether individual transgenic animals that express the transgene poorly, and hence are substantially equivalent to their non-transgenic relatives, can be safely introduced into the food chain.

The review covers the techniques currently being used to produce transgenic animals, the techniques that might be used to trace transgenes in animals and the products derived from them and finally we discuss some issues associated with stability of the introduced transgenes. Whenever reasonable, we present predictions about future developments in the field of food animal transgenesis.

## Context and background

This review was commissioned by Food Standards Australia New Zealand (FSANZ) with the specific goals and objectives defined in Appendix 2.

From a scientist's perspective, the global field of eukaryotic transgenesis has been particularly exciting, as the techniques allow biological scientists to probe the functional relationships between genes, gene products, developmental structures and animal phenotypes. It has been suggested that the vast majority of transgenic animals are mice and that the money spent on creating them is in the order of $A200M yearly (Marcia Ward *pers. comm*.). Notwithstanding the direct significance of transgenic mice to the advancement of biological science, and indirect significance to animal production science, the focus of this review will be transgenic animals that could be the source of human foods or nutriceuticals.

From a broader perspective, transgenics in general (genetically modified organisms - GMOs), and transgenic animals in particular, have generated considerable controversy in the developed world. The opportunities and threats raised by this rapidly developing technology have challenged policy makers to extend regulatory, animal welfare, food safety, environmental and ethical frameworks to accommodate a range of previously unheard of possibilities. As a response to this controversy, governments have commissioned a number of reviews of transgenic animals and these have been a valuable source of information for the review team (Royal Society report; Committee on Defining Science-Based Concerns Associated with Products from Animal Biotechnology 2002).

Although Australian and New Zealand researchers are actively involved in the development of transgenic animals, the major proportion of all transgenic animals are developed outside these countries. Hence, in addressing the prospective aspects of the review (1a), the team reasoned that many of the drivers for transgenic animal development in Australia and New Zealand over the next five years currently exist outside these two countries. Hence we have investigated and documented developing fields of animal transgenesis globally, and have attempted to relate those developments back to the Australian and New Zealand animal food production systems.

## Limits of the review

The limits of this review were set as terms of reference in the contract. We note that these terms excluded some issues that might arise due to the development or application of transgenic technologies in the food animal production industry. This includes regulatory issues concerning the release of transgenic animals into the wild, or indeed their use in standard agricultural systems.

The review does not describe the application of cloning technologies to food animal production, as we understand this has been covered by a separate review. It deals with nuclear transfer technologies only in so far as they relate to development and propagation of transgenic production animals.

The review does not cover recent developments in the field of small regulatory RNAs. Whilst potentially very important in the aquaculture species (and eventually in other species), experimental use of these RNAs may lead to phenotypes that are not heritable across generations.

The likelihood of adoption and potential economic benefit of adoption in the animal production industries in Australia and New Zealand were not considered in the review.

The review does not systematically identify, or attempt to quantify, risks associated with development or application of transgenic technologies or the release of transgenic animals into the environment. Only in circumstances where the review team wished to highlight potentially adverse events that might not be immediately apparent to the reader, does the review cover the likelihood of these events.

Consistent with the statements that "a food produced using gene technology means a food which has been derived or developed from an organism which has been modified by gene technology" and that a "gene technology" is defined as meaning "recombinant DNA techniques that alter the heritable genetic material of living cells or organisms", the team has excluded from its consideration growth promotants produced in transgenic microbial expression systems.

The review considers only briefly the use of transgenic animals as xenotransplantation donors in its analysis. We refer interested readers to the reports of the Royal Society (2001) and the US National Research Council (Committee on Defining Science-Based Concerns Associated with Products from Animal Biotechnology 2002) where the potential risks of such animals entering the human food chain are thoroughly described.

Of necessity, this review has been limited to information readily available in the public domain together with information that our survey respondents were prepared to allow into the public domain (see Survey methods below). The reader should be aware that there is likely to be a good deal of information relating to relevant technologies under development in private and public research establishments that has not been published, in either the scientific or the patenting literature. Indeed, the authors' enquiries were limited on several occasions by commercial-in-confidence arrangements. In this context, it is of interest that we have had occasion to cite only 12 patents in contrast to more than 100 papers in the open literature.

We have addressed neither the ethics of the use of transgenic animals in food production nor the ethics of animal experimentation. Though relevant to all considerations of gene technology in livestock production they do not come within the terms of the review.

## Survey methods

The review team used two methods to collect information. The first involved computer-based search strategies. Primary contributors conducted a literature review within their field of expertise and added key, cited literature to a central database. The principal databases used were: Medline (PubMed); CAB Abstracts; AGRICOLA; Science Citation Index (Web of Science); Current Contents; Biological Sciences (CSA) and Derwent World Patents Index (Derwent Innovation Index). Supplementary searches to identify current research were conducted in Zoological Record; NTIS (US); Australian Bibliography of Agriculture; Australian Rural Research in Progress; Current Agricultural Research Information System (FAO); Current Research Information System (USDA)

To obtain an overview of current regulatory and policy developments, Internet searches were conducted using Google and Scirus, with supplementary searches across Australian and international library catalogues. WWW sites of relevant regulatory bodies in Australia, New Zealand, USA, Canada, UK and the EU were also examined.

Current news covering scientific, social and political developments and issues was reviewed using AgBioTechNet and DIALOG Newsroom.

The second method involved consulting our professional contacts. Table 1 contains the names and contact details for the people we contacted. Each person was asked a standard series of questions relating to the central questions of the consultancy. Approximately half of these people provided useful information, but all provided at least one lead onto another information source. Generally, the reliability of the information was not questioned, though corroborating evidence was certainly incorporated into the review, when available. The people contacted received no direct remuneration or benefit from contributing to the review. When asked specifically we provided our understanding of the goals of the FSANZ in commissioning this review.

# The transgenic food animal field

The primary goals of food animal production have not changed radically for half a century despite the emergence of transforming technologies such as transgenesis. These are the efficient and humane production of safe, nutritious and enjoyable foods, without significant degradation of the natural resource base. Transgenesis has opened a number of new opportunities to increase the nutritional qualities of the animal foods, as well as the use of the food animal, or more specifically its organs, as factories for production of nutrients and therapeutic proteins.

Apart from production of pharmaceuticals, the targets of animal transgenesis have mostly been the same targets as for quantitative genetics and selective breeding: efficient production (feed conversion efficiency, disease or parasite resistance, growth rate under normal production conditions), food safety (resistance to transmissible spongiform encephalopathies (TSEs), where worrying results have recently been published; see Houston *et al*., 2003) and nutritiousness and wholesomeness (modified fatty acid profile). The concept being that genetic progress will be more rapid once the genes responsible for population variation in a particular trait can be manipulated directly, provided that pleiotropy and epistasis are not overwhelming. There are no examples at this time of transgenic animals designed to address two other traditional targets of breeding: eating enjoyment (flavour, tenderness or juiciness); or fitness for industrial purpose (size and shape of the product as one observes in fruit breeding). We found one example of a transgenic animal species designed to grow in environments more extreme than would be normal for the particular species (freeze-resistant salmon). Also, there is one example of a transgenic pig designed to reduce the offsite impacts of intensive pig production (Enviropig). Generally then, transgenesis in animals appears to be focussed more on performance under normal production conditions.

With the exception of farm animals used for pharmaceutical production in milk there are no transgenic farm animals in commercial production anywhere in the world and, even if given regulatory approval, it is likely to be many years before their appearance in the market. Transgenic carp and salmon are arguably the closest organisms to commercialization (Table 3). Nonetheless many types of gene introduction have been attempted or are in train, and methods for production continue to be improved.

# Definition of the animals and the products derived from them

## Terrestrial animal transgenics

A detailed list of the transgenes that have been applied in livestock is given in Houdebine (2002). Our searches revealed a number of transgenic experiments on five livestock species (Table 2), as well as a number of developments in mouse that could conceivably flow onto livestock species. Where possible, we have summarised information about the animals and the methods used to characterise their phenotypes. In other cases we provided the key references. We have also provided a list of compositional data from transgenic livestock and aquaculture species based upon published results (Table 4).

There has also been significant activity in the development of transgenic animals for pharmaceuticals, biomaterials and for xenotransplantation (Table 2). In number, these animals clearly outweigh the animals developed with agricultural applications in mind. The production and use of such transgenic animals falls under the regulatory jurisdiction of the Office of the Gene Technology Regulatory (OGTR) in Australia, and the Environmental Risk Management Authority (ERMA) in New Zealand, before they would become of regulatory interest to Food Standards Australia New Zealand (FSANZ) or the Australia and New Zealand Food Regulation Ministerial Council (ANZFRMC). The authors could imagine circumstances where unwanted transgenic animals might be considered for delivery into the food chain, and all elements of the regulatory process would need to be involved in the assessment of safety of this delivery. Examples would be the bull calves or cockerels produced from creation of a transgenic animal expressing proteins from another species (heterologous protein

expression). The male animals will express significantly less of the protein of interest, and hence will not be economically valuable. Likewise female animals that are low-expressors of the recombinant product (for some ill-defined reason), might be discarded into the human food chain intentionally or as an over-sight.

## Transgenics in the aquaculture industries

Aquaculture species have been particularly amenable to the production of transgenics. Fish and shellfish tend to be highly fecund, producing a large quantity of gametes. Many species can be harvested for eggs and sperm and fertilisation *in-vitro* is often straightforward. Eggs are relatively large, and fertilised eggs tend to develop outside the body, so no further manipulation, such as re-implantation is necessary.

The first successful gene transfer experiment in fish occurred in 1985 in China (Zhu *et al.* 1985). A DNA construct consisting of human growth hormone under control of the mouse metallothionein promoter was injected into the germinal disc of an early-stage goldfish *Carassius auratus* embryo. Microinjection procedures were quickly perfected by other groups in Norway (Rokkones *et al.* 1985), France (Chourrout *et al.* 1986) and Japan (Ozato *et al.* 1986). Brem *et al.* (1988) were among the first to produce a commercially important fish (Nile tilapia *Oreochromis niloticus*) bearing a human growth hormone transgene, again under the control of the mouse metallothionein promoter. Since this time transgenics have been generated in a number of commercially important species (Table 3).

Invertebrates such as crustaceans and bivalves are also amenable to transgenic techniques. The technology has lagged behind that of teleost fish however because of the absence of suitable DNA sequence information and problems in closing the life cycle of many species. Transient or single generation expression of reporter constructs has been demonstrated in the Kuruma prawn *Pernaeus japonicus* (Preston *et al.* 2000), the black tiger prawn *Pernaeus monodon* (Sulaiman *et al.* 1999, Tseng *et al.* 2000), giant freshwater prawn *Macrobrachium rosenbergii* (Li & Tsai 2000), red swamp crayfish *Procambarus clarkii* (Sarmasik *et al.* 2001), red abalone *Haliotis rufescens* (Powers *et al.* 1995), Eastern Oyster *Crassostrea virginica* (Cadoret *et al.* 1997, Buchanan *et al.* 2001) and dwarf surfclams *Mulinia lateralis* (Lu *et al.* 1996). To date, only one phenotypically relevant, stable shellfish line has been produced. Tsai *et al.* (2000) engineered a line of Japanese abalone *Haliotis divorsicolor suportexta* which express Chinook salmon growth hormone. The animals were reportedly faster growing than their non-transgenic conspecifics. However questions remain about the appropriateness of the construct in a gastropod system. Research into crustacean and molluscan transgenics is a potentially lucrative area, and advances in culture technology coupled with the increasing volume of gene sequence information will undoubtedly prove beneficial. In addition to the academic research in this field, Farming Intelligene, a Taiwanese-based biotechnology company is currently working to develop a transgenic line of Pacific white shrimp *Litopenaeus vannamei*, although current progress is confidential.

### Transgene construct design

Early studies on gene transfer in fish utilized mammalian and avian coding sequences, due to the lack of suitable piscine alternatives (Iyengar *et al.* 1996). However, concerns remained about the appropriateness of the constructs in piscine systems, particularly where intronic regions were present (Friedenreich & Schartl, 1990, Betancourt *et al.* 1993, Iyengar *et al.* 1996). The promoter sequences used in the constructs were often viral, and whilst these tended to produce a high level of expression, again, questions remained about the appropriateness of the sequence, and it was clear that they would never be acceptable for food use. In the 1990's however, a greater number of piscine genes were sequenced, in particular those encoding growth hormone. The cloning of the β–actin promoter from carp allowed the production of an all-fish construct (Liu *et al.* 1990).

### Promoter choice

Several promoters have been examined for their ability to express genes in fish (summarised in Devlin 1998). Metallothionein promoters were among the first eukaryotic promoters to be used in fish.

Metallothioneins are proteins that bind heavy metals in cells, in particular cadmium, copper, zinc and mercury (Maclean & Penman 1990). They function to supply zinc to zinc-requiring enzymes within the cell, and in detoxification. Metallothioneins are inducible, and their synthesis is up-regulated in the presence of heavy metals. Thus, the promoter sequences of the metallothionein genes may be used to regulate transgene expression in experimental animals, in response to heavy metal administration. Initially, the mammalian sequences e.g. mouse (Zhu *et al.* 1985) were used, but later fish specific sequences were cloned (e.g. rainbow trout, Zahfarullah *et al.* 1989). However, the necessity of heavy metal induction for expression obviously rules them out for use in food fish. Viral promoters or human gene promoters are also ill-favoured due to concerns about their public acceptance in foods (Du *et al.* 1992). However, two other promoters have shown much more promise: the β–actin promoter and the antifreeze protein (AFP) promoter. β-actin and its promoter are evolutionarily conserved sequences, and the promoter can be used to drive ubiquitous tissue expression. The rat β–actin promoter was the first to be used successfully in a construct, but later the carp and tilapia sequences were identified. The ocean pout *Macrozoarces americanus* AFP promoter has also shown promise in Canadian trials with pacific salmon. In general, it is likely that species-specific promoters will give the best expression. For instance trials with the tilapia β–actin promoter fused to the β-galactosidase coding sequence (as an experimental reporter of gene expression) have shown significantly higher expression in transgenic tilapia than the carp β–actin promoter (Hwang *et al.* 2003).

**Choice of expressed gene.**

The choice of coding sequence for expression depends upon the trait to be modified. Most attention has focussed upon genes that accelerate growth, but other traits such as disease resistance have been investigated. In addition, reporter genes, the products of which are easily detectable in the host, have become increasingly used for feasibility studies on new species, new delivery methods or new promoter sequences.

Among the first attempts at producing a commercially viable fish for the aquaculture market were attempts to produce a "freeze resistant" Atlantic salmon strain that would extend the potential range of aquaculture operations on the east coast of Canada (Fletcher *et al.* 2000). The construct used was made from the promoter and coding sequence of the ocean pout AFP gene. AFPs are small peptides produced by a number of marine teleosts that inhabit waters at subzero (0 to –1.8ºC) temperatures. The peptides serve to lower the freezing point of the blood plasma, protecting the fish from freezing. Many commercially important fish species (such as salmon) lack AFP genes and, as a consequence will not survive sub-zero seawater temperatures. However, the levels of AFP produced by the transgenics were too low to confer any significant freeze resistance in the strain (Fletcher *et al.* 2000).

Of all of the transgenic aquatic production species that have been generated, growth hormone (GH) transgenics account for a far greater proportion than all the others together. Indeed, the first transgenic fish ever made was a GH transgenic goldfish (Zhu *et al.* 1985). GH is a polypeptide synthesized in the anterior portion of the pituitary glands of all vertebrates (Maclean & Penman 1990), under the control of the central nervous system (CNS). From the pituitary gland it is released into the circulation to stimulate growth and development. In order to bypass CNS control and modify expression levels it is necessary to modify the tissue-specific regulatory elements of the gene. Most early experiments on these constructs utilised mammalian, viral or avian promoter sequences, but poor expression was frequently observed (Guyomard *et al.* 1989, Penman *et al.* 1991, Houdebine & Chourrout, 1991). Later however, the Chinook salmon GH gene was fused to the ocean pout AFP promoter to produce an "all fish" construct. When transformed into various species of salmonids, expression was predominantly in the liver, but elevated GH levels occurred in the blood stream (Fletcher *et al.* 2000). Other regulatory sequences such as the carp β-actin promoter were later utilised providing a ubiquitous and high level of expression (Alam *et al.* 1996). Most recently, a South Korean group (Nam *et al.* 2001) have achieved "autotransgenesis" in the mud loach *Misgurnua mizolepis*, a commercially important species on Asian markets. The construct consists of the mud loach β-actin promoter fused to the loach GH coding sequence. The construct has been used to generate three

strains of "auto-transgenic" mud-loach, by stable incorporation into the germ line. The resulting strains exhibit between 2- and 35-fold greater growth rate than the non-transgenic controls, and the time to attain marketable size is only 30-50 days post-fertilization, rather than 6 months.

Many species have shown increases in growth rate upon introduction of a GH transgene (Table 3). However, Devlin *et al.* (1995) achieved particularly striking results. Coho salmon *Oncorhynchus kisutch* transgenic for an AFP-GH construct, showed an 11-fold (average) difference in weight compared to the controls 15 months post-fertilisation. The largest fish in this study reached a weight 30-fold higher than that of the controls. These fish do not grow beyond the normal adult size finally, but their accelerated growth rate allows them to reach market size in 2 rather than 3 years. Devlin *et al.* (2001) took a strain of slow growing wild rainbow trout *Oncorhynchus mykiss* and a strain of fast growing domesticated rainbow trout and introduced to each a construct consisting of the rainbow trout metallothionein promoter fused to the Chinook salmon growth hormone sequence. Whilst the wild strain exhibited an average 17.3-fold difference in weight by 14 months post-fertilization between transgenic and non-transgenic fish, there was no difference in weight between the transgenic and non-transgenic domesticated strain. In addition, the growth of the transgenic wild strain did not surpass that of the non-transgenic, fast-growing domesticated strain. These results indicate that the potential for growth enhancement is strongly dependant upon genetic background and, in some cases at least, similar alterations in growth rate can be achieved both by selection and transgenesis, but that the effects are not always additive, let alone synergistic. It has also been noted that differences in growth enhancement between transgenic lines derived from the same strain can arise from differences in the chromosomal site of integration of the transgene and the number of tandem inserts (Zhu *et al.* 1992). A competitor for the first commercial transgenic food animal is the Yellow River Carp produced in China by Prof. Zhu and his team at the CAS Institute of Hydrobiology. We have found numerous news articles and anecdotal reports about this development (e.g. http://www.bulletin.ac.cn/ACTION/2000102601.htm; Pew Initiative report (2003)), but no rigorous, peer reviewed information.

Pitkanen *et al.* (1999) tried an approach to growth promotion different from transfection with the GH construct. The premise was that most farmed fish are carnivorous, and that the fin-fish aquaculture industry depends upon a relatively expensive supply of fish oil in the feed pellets. They tried to improve carbohydrate metabolism using the human glucose transporter type 1, and the rat hexokinase type II genes in rainbow trout and Arctic charr *Salvelinus alpinus*. Expression of both constructs was demonstrated in founder animals but unfortunately the results were inconclusive because of mosaicism.

In intensive culture, fish are susceptible to bacterial and ectoparasitic infection. Hence, constructs which induce an immunostimulatory response or which encode anti-pathogenic products might be of benefit to the aquaculture industry, in terms of lower losses from infections, and a reduced requirement for treatment and/or vaccination. Dunham *et al.* (2002) produced a channel catfish containing the gene for cecropin, an antibacterial peptide originally isolated from the moth *Hyalophora cecropia* (Steiner *et al.* 1981). The fish were challenged with the pathogenic bacterium *Flavobacterium columnare*. During the resulting epizootic event, 100% of individuals containing a preprocecopin B construct survived, whereas 27.3% of non-transgenic controls survived. ($P<0.005$). In a parallel challenge study, individuals carrying a catfish immunoglobulin leader/cecropin B construct were challenged with *Edwardsiella ictaluri* which causes enteric septicemia. In this case 40.7% of transgenic individuals survived versus 14.8% of controls ($p < 0.01$). Neither of these transgenes was shown to influence growth rate.

## Methods of achieving sterility of transgenic fish/shellfish

Methods of inducing sterility in transgenic fish are important for two reasons, firstly the escape of transgenic fish or shellfish from the seacage environment is of real environmental concern, and has motivated researchers to develop strategies to render escapees sterile. Secondly, any company

marketing a transgenic strain would benefit from selling only sterile individuals, thereby preventing others from growing and maintaining their own broodstock.

Chromosome set manipulation is one well-established method of achieving this, through ploidy manipulations and/or manipulation of the sex chromosomes (though strictly speaking this is not a transgenic technique). Triploidy or tetraploidy can be induced in fish and shellfish eggs by applying a pressure, temperature or chemical shock shortly after normal fertilization, which prevents extrusion of the second polar body. Triploids are generally sterile, providing a level of "reproductive containment" (Lyons & Li 2002). However, triploidy does not ensure a 100% sterility rate. Success rates vary between 10 and 95% (Maclean *et al.* 2002). Triploidy can also decrease growth performance and survival during the maturation phase (Withler *et al.* 1995), although post-maturation somatic growth can be higher due to a lower proportion of resources being directed into reproductive growth (Wolters *et al.* 1982). Razak *et al.* (1999) has produced the most convincing results on reproductive sterility to date. Triploidy was induced in growth hormone transgenic tilapia by heat shock. The triploid males exhibited significantly smaller testes, although spermatozoa were still present. However the females produced ovaries that were devoid of oocytes and were completely non-functional.

Recently, Uzbekova *et al.* (2000) pioneered an RNAi approach, attempting to silence the gonadotropin-releasing hormone (GnRH) gene in rainbow trout, which is the main neuromediator controlling the gonadotrophic function of the brain pituitary axis. However, whilst maturation of the fish containing the construct was more asynchronous, there was no significant difference in the timing of maturation or in the proportion of fish reaching maturation.

## Pleiotropic Effects

When a construct is inserted into an organism with the objective of improving a specific trait, that construct may affect more than one phenotypic character. These "pleiotropic effects" may be positive or negative (Dunham & Devlin 1999). For instance, Chatakondi *et al.* (1995) found that common carp transgenic for the rainbow trout growth hormone had a positive influence on survival from the fingerling size upwards, when subjected to a series of stressors and pathogens such as low oxygen, anchor worms and dropsy. Growth hormone has important physiological effects on energy absorption and utilisation in vertebrates, which is manifest in the altered body compositions found in some transgenic fish relative to controls (Figure 4). Differences in the rate of growth may be attributed to changes in several factors; gross feed intake, feed conversion efficiency, faecal loss or protein turnover. GH also stimulates the synthesis of protein over fat, so the ratios of protein/lipid have been shown on a number of occasions to be higher in transgenics than the controls (Arctic charr, Pitkanen *et al.* 1999; common carp, Chatakondi *et al.* 1995, Fu *et al.* 1998, 2000, Dunham *et al.* 2002, Martinez *et al.* 2000). Most strikingly, GH transgenic silver seabream *Sparus sarba* exhibited a 50% decrease in fillet fat content (Lu *et al.* 2002). This change in protein:lipid ratio has been touted as a health benefit (Chatakondi 1995), but the real impact on the complex parameter of "flesh quality" in terms of consumer choice is unknown. However, the amino acid and fatty acid profiles have been shown to be extremely similar between transgenic and non-transgenic strains (Martinez *et al.* 1999, Fu *et al.* 2000, Dunham *et al.* 2002).

In carp and salmon, body shape has been seen to change as a result of GH transgenesis. Transgenic carp tend to have deeper and wider bodies and larger heads. This is manifest in a higher dressing percentage in transgenic fish (Dunham *et al.* 2002). Again, the authors report the subjective impression that the transgenic fish had a "better quality of flesh". The change in head and body dimensions is most extreme in GH transgenic coho salmon, the most rapidly growing individuals of which show some morphological disruptions of the cranium analogous to acromegaly syndromes observed in mammals overexpressing GH (Devlin *et al.* 1995). These morphological disruptions in some individuals became quite severe and have been seen to affect respiration, feeding, growth rate and ultimately viability. Similar although less pronounced observations were made on GH transgenic tilapia by Rahman *et al.* (1998). In these fish, all transgenic males also exhibited reduced or non-

existent sperm production, likely due to differential resource partitioning away from reproductive growth.

## Methods for the Production of Transgenic Livestock

The production of transgenic livestock is still at the laboratory scale, and until the first transgenic food animal has passed through the regulatory systems, there will be no incentive for industrialists to invest in increasing the scale of the operations. Whilst there are techniques that have been utilised to generate transgenic animals, especially a large number of methods for the mouse, none have been reliable and applicable across a broad range of species.

Two fundamental determinants of the success of any transgenic manipulation of a livestock species, are the transport of DNA across the plasma membrane of the recipient cell, and the transport of that DNA across the nuclear membrane to gain access to the chromosomes. A third determinant is the incorporation of the transgenic DNA into the chromosome to allow germline transmission and controlled stable expression of the new gene. The techniques described below all have aspects that address these three determinants in different ways. A number of reviews have been published recently and these describe the commonly used methods (Wall 2002; Niemann and Kues 2000; Houdebine 2002). This section of the review seeks to build on their comments and update the available information, and is summarized in Table 6.

### Nuclear Transfer

Nuclear transfer is the transfer of the nucleus from a selected cell into an enucleated single cell embryo. For transgenesis, this technique allows the genetic manipulation of a cell line and transfer of the nucleus to an enucleated embryo to generate a transgenic animal, avoiding some of the inefficiencies associated with other methods. Nuclear transfer *per se* remains technically difficult and requires specialised equipment, but advances are being made in manipulation of the cells and the types of cells that can be used as nuclear donors (Wilmut *et al.* 2002). The most commonly used cell types are fibroblasts and embryonic stem cells. Recently the technique of whole cell injection has been used to generate a recombinant embryo (Lee *et al.* 2003). An enucleated embryo was injected with an

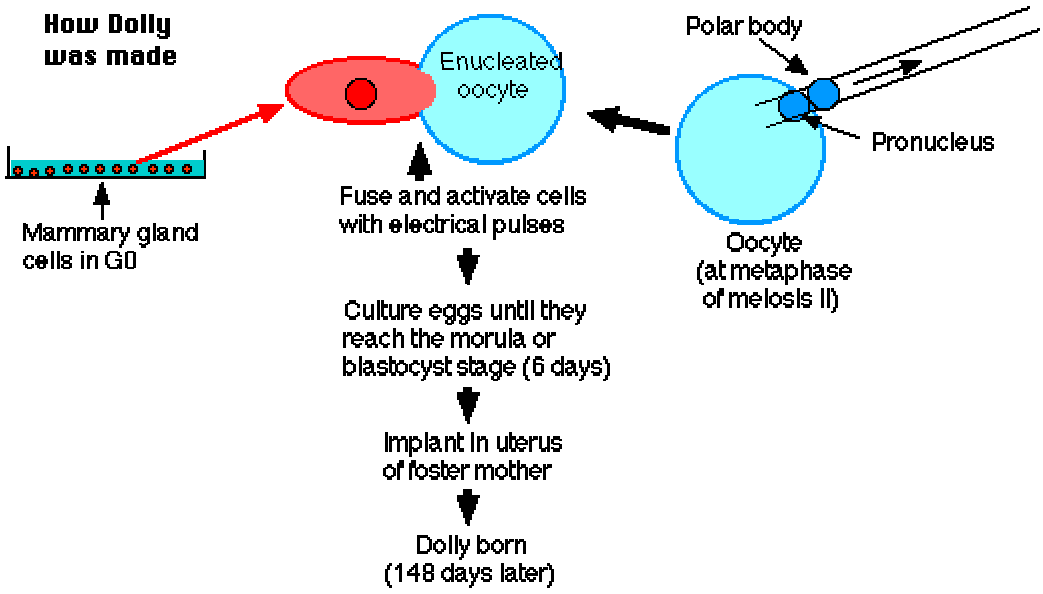


**Fig. 1. Schematic representing nuclear transfer**
For the generation of transgenic animals the parent cell from which the nucleus is derived would be a genetically engineered cell line not the mammary cells as depicted.
(Source: http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/CloningMammals.html#why_not).

intact somatic cell resulting in a viable embryo. This technique results in similar percentages of transgenic embryos as for standard techniques, but is easier and requires less specialised equipment, making this technology available to more labs around the world.

Nuclear transfer often results in high birth weights and physical abnormalities (Wilmut and Paterson 2003) (Dinnyes *et al.* 2002; Schrader *et al.* 2003), which along with its inefficiency, might limit the broad application of this technique.

## Microinjection

Microinjection is the standard method for introducing DNA constructs into fin-fish. In most species the pronulei are not easily visible, so injection is into the cytoplasm. In addition, the vitelline membrane is surrounded by a tough chorion. In some species direct injection through the chorion into the blastodisc is possible (channel catfish *Ictalurus punctatus*, Dunham *et al*. 1987; common carp *Cyprinus carpio*, Zhang *et al*. 1990; northern pike *Esox lucius*, Gross *et al*. 1992). In other species however, injection is performed through the micropyle - a tiny pore in the chorion through which the sperm penetrates. Other researchers have treated eggs with reduced glutathione, which prevents chorion hardening post fertilisation (Yoshizaki *et al*. 1991).

Microinjection of embryos with DNA has been the traditional approach for generating transgenic livestock. Microinjection is the physical injection of a solution of DNA into the pronuclei of a zygote. The method relies on random integration of the transgenic DNA via the recruitment of cellular DNA repair pathways and remains a highly inefficient process with success rates of only 1-4% (Niemann and Kues 2000). The cellular repair pathways can also fragment the DNA, separating coding regions from promoters prior to integration (Murnane *et al*. 1990). The inefficiency of this process is costly and time consuming with estimates of $500,000 (US) to generate a cow that is expressing a transgene (Wall *et al.* 1992). In addition there is no control over the site of insertion or the number of copies of the transgene inserted in the genome, both of which can vary greatly between individuals from the same experiment. The site of insertion can have a significant effect on the level of expression of the transgene and multiple copies of the gene will increase the chances that normal gene function is altered as more genes are interrupted by insertion. Mosaicism is also a problem. The majority of animals generated by microinjection are mosaic (Whitelaw *et al.* 1993) and require a second generation screening process to ensure that the transgene is incorporated into the germline.

Despite all these drawbacks the process is still being used to generate transgenic animals (Baldassarre *et al.* 2003; Behboodi *et al.* 2001) and is being improved upon by the use of such techniques as the co-injection of restriction enzymes with the DNA to mediate incorporation of the transgene into the chromosome (Thermes *et al.* 2002).

## Sperm mediated gene transfer

An alternative approach to microinjection is the use of sperm to deliver the transgenic material to the oocyte during fertilisation. Initial experiments have involved incubating the sperm with the DNA then using *in vitro* fertilisation techniques to transfer the DNA into the oocyte during fertilisation. This approach has previously proven inefficient and inconsistent (Gandolfi 2000). Recently, research has been carried out to determine the appropriate conditions to use when incubating DNA with sperm (Lavitrano *et al.* 2003). This was done to get maximum uptake and ensure efficient production of transgenic pigs with efficiencies as high as 50% - 60% being recorded. Effects of cell cycle and the quality of sperm of different sires all contribute to the overall success, but the method is now being optimistically percieved as a valuable technique for transgenic animal production.

To increase the efficiency of sperm uptake of DNA various approaches are being taken. One is to attach the recombinant DNA to the sperm head via an antibody fused to the DNA (Chang *et al.* 2002). The antibody used in this work recognises surface proteins common to sperm from cattle, pigs, sheep, chicken, goats, mice and humans. This approach ensures that the DNA remains associated with the sperm during fertilisation. In consequence, perhaps, reported efficiencies are high with up to 37.5% of

the progeny being transgenic when this process is used to generate transgenic pigs. Another approach has been to place the DNA inside the sperm head by electroporation (Rieth *et al.* 2000) or lipofection (Lai *et al.* 2001) and both these methods have resulted in the generation of transgenic progeny. Injection of DNA into the testis is another approach used to transferring the DNA to sperm. This method has the advantage that there is no manipulation of the sperm and fertilisation occurs via natural means (Celebi *et al.* 2003). With the improvement in techniques for culturing and expanding spermatogonial stem cells there is now also the possibility of engineering these cells in-vitro to generate transgenic sperm that could be used to fertilise oocytes and generate transgenic animals (Brinster 2002; Nagano *et al.* 2000).

## Homologous recombination

Homologous recombination is used generally to delete a gene. The most common approach is to make a construct that interrupts the gene of interest with a marker protein such as the green fluorescent protein. The cells/embryos in which recombination has occurred are selected by the presence of the marker protein. This method may have a small but limited use in applications for transgenic livestock as usually researchers seek to add genes in not remove them. However, gene deletion may be productive for manipulating expression in metabolic pathways controlled by negative regulator genes (e.g. myostatin in muscle), and has been used to delete the prion gene in an experimental sheep (Denning et al. 2001).

One approach that could prove useful is the use of chimeraplasts to generate single base pair alterations in targeted genes. This may be useful in introducing a single nucleotide polymorphism (SNP) such as that associated with the callipyge locus (Smit *et al.* 2003). Chimeraplasts are DNA /RNA hybrids that are very active in homologous pairing reactions (Stephenson 1999). Chimeraplasts have been used to correct a number of genetic diseases both *in-vitro* and *in-vivo* and show great promise (Bartlett *et al.* 2000; Rando 2002). There are concerns about the ability of the constructs to enter the nucleus and recombine with chromosomal DNA, and recent work suggests good efficiency in alteration of episomally located DNA but no transformation of chromosomal DNA (Tran *et al.* 2003). This severely limits its usefulness in the generation of germ line transformants though continuing studies into the mechanisms of nuclear homing (Chan and Jans 2001; Liang *et al.* 2000) may resolve this difficulty.

Other approaches to homologous recombination include the use of ALU-like repeat sequences that increase the efficiency of DNA recombination. This approach has been combined with electroporation of sperm to improve efficiency of transgenic animal production (Rieth *et al.* 2000).

## Transposon mediated gene transfer

Transposons are segments of DNA that can become integrated at many different sites along a chromosome. These DNA sequences code for a transposase enzyme that enables integration of the DNA into the host's chromosome. They have been demonstrated in bacteria as well as plants and insects, and they may also exist in crustaceans (Sigrid Lehnert pers. comm..). In vertebrates *tcl*-like transposable elements are found integrated into genomes, though they appear to be inactive (Lohe *et al.* 1995). Transposons have been used to generate mutant and transgenic *Drosophila* for some time (Kimura 2001) but now the opportunity exists to use these types of vectors to generate transgenic mammals. Ivics *et al* have reconstructed a transposable element from fish that is based upon a *tcl*-like transposon (Ivics *et al.* 1997). This transposon has been termed *Sleeping Beauty* and has been used to integrate DNA into human and mouse embryonic stem cells (Cooper 1998). More recently Harris and colleagues have refined this transposon into a single plasmid that they have called *Prince Charming* (Harris *et al.* 2002). The advantage of this transposon is its ease of use in generating site-specific integration of transgenic DNA. Whilst this vector system has only been used in cell culture, stability in long-term culture of cells has been demonstrated and this method should be easily applied to the generation of transgenic somatic or stem cell lines that could then be used in nuclear transfer.

## Retrovirus-mediated gene transfer

Retroviruses are nature's natural gene delivery system. In a single protein package comes nucleic acid that can redirect a cell's synthetic machinery to express the viral genes as well incorporate the viral genome into the host cell genome. Retroviruses are being explored extensively for use in human gene therapy and have been used in a clinical situation to treat genetic diseases (Thomas *et al.* 2003). Attempts have also been made to use these vectors to engineer transgenic animals though with little success (Baltimore *et al.* 2003). This has been attributed to the limited cell host range of the vectors used.

Recently lentivirus constructs have been made and used to infect embryonic tissue resulting in the generation of transgenic rats and mice (Rubinson *et al.* 2003). These vectors have a significant range of cell types that they can infect making them applicable to the generation of transformed somatic or stem cells for use in nuclear transfer. Thus there now exists retroviral vectors that can make the generation of transgenic animals much more efficient.

Other advances in the use of retroviral vectors include injection of the retrovirus into unfertilised embryos. Chan *et al*. (Chan *et al.* 1998) have described this technique and claim that the majority of offspring produced this way are transgenic. More recently small hair pin RNA molecules have been delivered to both cycling and non-cycling cells using a lentivirus construct (Rubinson *et al.* 2003) demonstrating the powerful combination of RNAi technology and retroviral mediated gene transfer.

Avian transgenesis has remained a notoriously inefficient procedure mainly because egg production and fertilisation is a very complex process in birds. Nonetheless, retroviral methods of modifying the chicken genome are progressing (Ivarie 2003).

Despite the promise of retroviruses for gene transfer in both gene therapy and the production of transgenic animals there are concerns about the use of these vectors. Issues of concern include the reactivation of the retrovirus causing a viral infection and the activation of oncogenes making the transgenic animal more susceptible to the development of tumours. The other disadvantage in using these vectors is the limited insert size that they can retain. This is an issue, as large inserts are often required to ensure stable regulated expression of a transgene.

## Artificial Chromosomes

Artificial chromosomes can carry extremely large DNA fragments (1 million bases - 1Mb or more). They are autonomous, self-regulating sequences possessing a centromere, two telomeres and origins of replication. Bacterial artificial chromosomes (BACS) have the capacity to carry up to 100kb, Yeast artificial chromosomes (YACS) can carry hundreds of kilobases, and mammalian artificial chromosomes (MACS) can carry Mb-size sequences. These artificial chromosomes can be used as transfer vectors that replicate autonomously in the cytoplasm of the host cell and are transmitted through the germline. Artificial chromosomes have recently been used to insert the entire human heavy and light chain immunoglobulin loci into cattle (Robl *et al.* 2003) and produce human polyclonal antibodies.

The main advantage of this technology is the ability to transfer large DNA constructs that ensure better control of transgene expression. The disadvantage is the difficulty in handling such large fragments of DNA and the fact that the chromosomes exist separately to the normal chromosomal complement of the cell; if the properties of insert survival could be controlled, this disadvantage might become an advantage.

## Techniques specific to the production of transgenic fish/ shellfish

*Electroporation*

Electroporation utilises short bursts of electrical current to temporarily render the vitelline membrane porous to macromolecules such as DNA. It is more suitable for the delivery of constructs to large numbers of eggs than microinjection. Electroporation of sperm or gonads prior to fertilization is also

possible and has been used successfully in the Chinook salmon *Oncorhynchus tshwytscha* (Sin *et al*. 1993). Electroporation is the favoured method in invertebrate transgenics.

*Particle Bombardment*
The delivery of DNA to fertilized eggs of loach, rainbow trout and zebrafish by particle bombardment was demonstrated by Zelenin *et al*. (1999). Plasmid covered tungsten microprojectiles were introduced at high velocity, resulting in the stable integration of reporter genes into the genome. However none of the embryos were grown to hatching. Similarly, Cadoret *et al*. (1997) introduced a luciferase reporter gene into embryos of the eastern oyster *Crassostrea virginica* using an almost identical technique. In this study embryos were not taken beyond the trochophore stage.

*Chemically mediated transfection*
Buchanan *et al*. (2001) tested a liposomally-mediated transfection technique similar to that used for mammalian cells in culture on eastern oyster embryos. Again, uptake of the reporter construct was demonstrated, but the resulting larvae were not taken beyond embryogenesis.

All techniques used to produce transgenic fish or shellfish (including microinjection) yield animals that are prone to mosaicism. If a construct is integrated into the genome at the 1-cell stage, all tissues will carry at least one copy of the novel sequence in each cell, which implies that the gonads will also be transgenic. If an animal is heterozygous with integration at a single locus, then 50% of sperm or eggs produced should carry the novel gene, and 50% of all progeny should be homozygous transgenic (Maclean & Penman 1990). However, if an organism has more than one copy inserted at different locations on different chromosomes, a complex ratio will be observed in the progeny, with >50% transgenism. However, due to the difficulty of pro-nuclear visualisation in fish and shellfish, constructs tend to be delivered to embryos that have already begun cleavage, and have often progressed as far as formation of the blastodisc. As a result, transgene mosaicism is an underlying feature of most founder individuals, and persistence of the construct in the organism does not guarantee inheritance to the next generation. Lower than expected ratios of transgene segregation to F1 progeny tends to occur, because in many cases the gene is expressed in a subset of somatic cells but not in the germline (Du *et al*. 1992; Devlin 1995; Fletcher *et al*. 2000).

## Markers

Whilst markers are not a method, this section is included to describe briefly the use of markers in the selection of transgenic animals/cells. The effectiveness of a new promoter, new species or new delivery mechanism can be assayed using a reporter system. The product of a reporter gene distinguishes cells that express the gene from those that do not. Commonly used reporter genes include the enzyme β-galactosidase (*Lac*Z), the product of which is detectable by histochemical staining, chloramphenicol acetyl transferase (CAT) and neomycin phosphotransferase (*neo*), which confer resistance to the antibiotics chloramphenicol and G418 respectively; luciferase which emits light in the presence of its substrates, and green fluorescent protein (GFP) which fluoresces under UV light (Devgan and Seshagiri 2003; Bordignon *et al.* 2003; Lai *et al.* 2002; Chan *et al.* 2002). Use of β-galactosidase as a visual marker for transgenic chickens, a difficult experimental subject, has been described (Mozdziak *et al*. 2003). The use of selectable markers for transgene integration in aquaculture species has been attempted but has met with little success (Powers *et al.* 1995, Cadoret *et al.* 1997, Tsai *et al.* 1997, Buchanan *et al.* 2001). This is most likely due to mosaicism within the embryo, where some cells exhibit resistance to the selection agent, but others are killed.

GFP has been used in transgenic cloned cows for preimplantation screening and to follow transgene expression in different generations derived from fibroblast cell nuclear transfer (Bordignon et al. 2003; Chen et al. 2002).

The use of particular markers may influence whether a food generated from the transgenic animals/cells is suitable for consumption. Richards and colleagues have tested the safety of the GFP

marker protein in food using a mouse model (Richards *et al.* 2003). They found that in their system there were no apparent adverse side effects from its consumption.

# Tracing transgenes

Molecular tools are currently the most cost-effective means of identification of transgenic animals and testing stability of the transgenes. A number of different techniques can be used for diagnostics and/or expression analysis, and will be discussed below.

## Transgene diagnostics

**Southern blot analysis**
Before the development of PCR (discussed below), Southern blotting was the method of choice for transgene detection (Table 7). It involves the isolation of DNA from selected animals, digestion of DNA with restriction enzymes, electrophoresis on agarose gels to separate DNA by size, denaturation of the DNA and transfer of the DNA to a membrane. This membrane (either nitrocellulose or more recently nylon) is then hybridised with a specific probe (complementary to the transgenic sequence of interest). The probe is usually radioactively, colorimetrically or chemiluminescently labelled such that once hybridised to the transgene DNA, the position of the transgene on the membrane can be visualised.
Southern blot analysis can be applied to:

- Demonstrating presence or absence of a transgene in the sample;
- Determining whether chromosomal integration has occurred;
- Identifying the position of integration relative to other samples;
- Determining whether homologous recombination (directed integration) occurred;
- Determining copy number of transgenes;
- Analysing stability of the transgene with regards to reintegration and/or and replication over time and through future generations i.e. the frequency of mutation/rearrangement within or adjacent to the transgene.

While no longer the method of choice for detection of transgenic DNA or transgene copy number, where PCR-driven methods have superseded it, Southern blotting analysis is still the best method for identifying positions of integration within the genome, and for analysis of the stability of integration over multiple generations.

**Standard polymerase chain reaction (PCR):**
PCR is an enzyme (polymerase)-driven method allowing the targeted amplification of specific regions of DNA from a sample. The method is rapid, giving diagnostic results in a matter of hours, and sensitive, requiring very small amounts of starting material (<1ng). PCR has applications in:

- Rapid screening (2-4hrs) for identification of transgenic animals;
- Detection of small amounts of transgenes in samples of blood or other tissues;
- Detection of mosaicism within an individual;
- Rapid assessment of environmental contamination / biosafety breaches.

Due to the ease of use, speed and sensitivity, standard PCR remains an important tool especially for identification of animals possessing the transgene of interest.

**Real-time quantitative PCR (Q-PCR)**
The real-time PCR system is based on the detection and quantification of a fluorescent reporter. The fluorescence signal increases in direct proportion to the amount of PCR product in a reaction (Heid *et al.,* 1996). This system has enormous application within the area of transgenics. Q-PCR has applications in:

- The same samples as for standard PCR;

- Automated, high-throughput screening of samples;
- Accurate quantification of the amount of transgenic DNA within a sample;

Accurate quantification of transgene copy number for monitoring genetic stability during production and breeding.

Q-PCR is likely to supersede standard PCR for most applications, because it is quantitative. Set-up costs of the technology may be prohibitively high for smaller laboratories however.

**Microscopy**

Microscopy can be used to identify transgenic vectors within cells. Fluorescence *in situ* hybridisation (FISH) is the most efficient cytogenetic molecular technique so far developed for the enumeration of chromosomes. Fluorescently-labelled probes for the transgenic sequence are used as probes to hybridise to chromosomes in which the transgene has integrated. These samples can then be enumerated using fluorescent microscopy. FISH analyses have been conducted on nuclear chromosomal DNA at various stages of cell division and on extended chromatin fibres (DNA fibre-FISH) and as well as naked DNA molecules. Once optimised, FISH can detect small and even single-copy transgenes within a genome. This technique has application in:

- Analysis of the chromosomal environment of integrated transgenes,
- Analysis of the local chromatin structure of transgenes,
- Assessment of the effect of integration position on gene expression (e.g. Dong *et al.,* 2001).

FISH and similar microscopic techniques will continue to be very important tools in the evaluation of transgene stability and expression.

**Transcription analysis**

As discussed below, stable expression of transgenes can be a major problem in generation of the desired transgenic animal. Methods of assessing expression are outlined below.

*Northern blotting*

Similar to Southern blotting in many respects (and hence the name), Northern blotting involves the isolation of RNA from selected animals or tissues, electrophoresis to separate transcripts by size and transfer to nylon membranes. This membrane is then hybridised with a specific probe (complementary to the expected expressed transgene transcript). The probe is usually radioactively, colorimetrically or chemiluminescently labelled such that once hybridised to the transgene DNA, the size and quantity of transcript can be visualised. By accurately loading similar amount of RNA per sample, it is possible to semi-quantitatively assess expression of the transgene in different tissues or animals. This technique has application in assessment of size and expression patterns of transcripts in different samples.

*Reverse Transcription PCR (RT-PCR)*

RT-PCR allows the generation of stable complementary DNA (cDNA) from RNA. The technique consists of two parts, synthesis of cDNA from RNA by reverse transcription (RT) and amplification of a specific cDNA by polymerase chain reaction (PCR). When applied to multiple samples it can be used in a comparative semi-quantitative fashion, or quantitatively using competitive RT-PCR. Absolute quantification, using competitive RT-PCR, measures the amount (e.g., $5.3 \times 10^5$ copies) of a specific mRNA sequence in a sample. Dilutions of a synthetic RNA (identical in sequence, but slightly shorter than the endogenous target) are added to sample RNA replicates and are co-amplified with the endogenous target. The PCR product from the endogenous transcript is then compared to the concentration curve created by the synthetic "competitor RNA." This technique has application in semi-quantitative or quantitative assessment of transgene expression in different animals or tissues. RT-PCR will remain a useful technique, but its use will decrease as the newer Q-PCR technologies become more accessible.

**Protein analysis**

While analysis of transcription levels is an important tool in identification of expressor animals, protein analysis quantifies the desired end product. DNA transcription without generation of the desired protein can be a problem in any experimental transgenic animal, and therefore demonstration of protein expression and function are the imperatives for successful transgenic studies. Methods used will vary depending upon the types of proteins being expressed.

*SDS-PAGE and Western blotting:*

Protein mixtures are isolated from tissues or by-products (e.g. milk) of interest, and these are separated based upon size or isoelectric point on polyacrylamide gels. This may be done in a one dimensional (size only) or two dimensional (size vs. isoelectric point) manner for increased separation. If over-expressed, no further analysis may be required, but the proteins can be transferred to membranes (Western blotting) and probed with antibodies to accurately identify the protein of interest or truncated products (non-functional). To use the technique one requires a highly specific antibody recognising one or more characteristic amino acid motifs within the protein of interest. This technique finds application in:

- Characterisation of protein expression in various animal tissues or tissue-derived products;
- Determination of molecular weight and isoelectric point of the product;
- Assessment of product solubility;
- Determination of quaternary structure (product complexation);
- Determination of the intracellular stability of the product (i.e. is the protein being rapidly degraded, as demonstrated by a range of smaller than expected products)
- Qualitative assessment of expression levels.

*Enzyme-Linked Immunosorbent Assay (ELISA)*

ELISA is a useful and powerful method for estimating protein concentrations down to the pg/ml range in biological fluids such as serum, urine, tissue homogenates or culture supernatants. Based on the principle of antibody-antibody interaction, this test allows for easy visualization of results. As with Western blotting, to use the technique one requires a highly specific antibody recognising one or more amino acid motifs within the protein of interest. This technique has application in the quantification of low concentrations of expressed product in transgenic animal tissues and or the products derived from these animals.

*Radio-Immuno Assay (RIA)*

RIA is similar in concept to ELISA, but is even more sensitive because it uses radio-isotope labelled tracer protein to quantify interaction with the protein of interest. It is mainly used to measure concentrations of hormone in plasma.

*High Performance Liquid Chromatography (HPLC)*

Liquid chromatography separates protein mixtures into their component molecules based on physical properties such as size, charge and hydrophobicity, or physicochemical properties such as ligand affinity. It is particularly valuable if the transgene product is well characterised, because it will allow verification of the equivalence of transgenic and non-transgenic gene products at the protein level. Coupled with protein N-terminal sequencing it can be used to completely define a protein's primary and secondary structure.

*Immunohistochemistry*

This is the technique for localising an expressed protein within cells and tissues, based on the affinity of specific antibodies. It is used to confirm the tissue specificity of protein expression, or to exclude the inappropriate accumulation of transgene products within cells and tissues. A further value of this technique would be observation of any structural malformation that might result from over-expression of a transgene, though in this case the technique would utilise antibodies to proteins other than the transgenic protein.

*Protein functionality*
It is axiomatic that many transgene products are functional proteins, and so confirmation of the function *in vitro* or *in vivo* is a key to understanding the impact to the animal of transgenesis. If the introduced transgenes encode an enzyme, it should be possible to demonstrate novel enzyme activity or in the case of "gene knockout" transgenesis, the loss of enzyme activity. Alternatively the introduced transgene might encode a structural protein and hence one should be able to demonstrate altered cell or tissue morphology or physiological function. An interesting example is the properties of the macrophages in transgenic pigs expressing human decay accelerating factor (hDAF). These were found to be resistant to lysis in the presence of human serum, demonstrating that hDAF is functional and confers resistance to human antibodies and complement (Lavitrano *et al.,* 2002)

Proof of functionality of transgene protein products demonstrates success of the experiment, and suggests that the transgene is stable. Continued assessment of function throughout the animal's life, and in future generations can also be seen as assessment of transgene stability over time. If the transgene is not stable, one would expect reduced or even eliminated function of the transgene product.

## Stability of Transgene expression

A major obstacle in the generation of transgenic livestock is that many introduced genes are expressed poorly or non-specifically. For example, Adams *et al,* (2002) reported that a growth hormone gene construct was not expressed in progeny of 1 of 3 rams tested. Expression was normal in the progeny of the other 2 rams.

In addition, there are documented cases of the transgene effect decreasing over time in the individual or in its offspring. An example of loss of the transgene effect is the transgenic insulin-like growth factor 1 (IGF-1) sheep generated by Damak and colleagues in 1996. First generation transgenic sheep had wool production rates up to 17% greater than non-transgenic sheep. However by the second season and in $F_2$ animals, no significant difference could be observed although the transgene was still present and widely expressed (Su *et al.,* 1998). It is possible that animals undergo metabolic adaptation to the presence of the additional IGF-1, as has been observed for injected IGF-1 (Lobley *et al*., 1998).

Positional effects in chromatin are deemed to be the major cause of expression loss. Enhancers and silencers in surrounding genes can activate or inactivate transgenes i.e. subtle interactions between the transgene and host's genomic DNA influence the expression of the foreign DNA (Cranston, 2001). A DNA methylation– directed histone deacetylation model has been proposed (Eden *et al.,* 1998; Razin, 1998). In this model, newly integrated DNA would be *de novo* methylated. This methylated DNA would then be a target of a protein complex which catalyses the deacetylation of histones in the region, leading to chromatin condensation and subsequent silencing of the gene. There is a growing body of evidence to support this model (Pannell *et al.,* 2000). The model is reminiscent of mechanisms of gene silencing for transposons/retroviral genomes, and as such it is no surprise that retroviral vectors are particularly prone to positional effects resulting in silencing. Recent evidence demonstrates that this methylation model does not completely account for silencing however, and other as yet unidentified factors, must also play a role (Pannell *et al.,* 2000).

A number of construct design factors have been shown to reduce transgene expression, and hence should be avoided. These are

- Use of small (less than 30kb length) transgene vectors;
- Use of cDNA used rather than genomic DNA with introns (If using cDNA, at least one intron included before cDNA is required);
- Integration of multiple copies of the transgene;
- Use of a bacterial gene;
- Presence of silencer elements (DNA-binding sites for *trans*-acting factors) that directly or indirectly reduce transcriptional initiation at promoters (Hilberg *et al.,* 1987).

There have been several approaches to minimising the silencing of transgenes and ectopic expression and these are outlined below. Any or all of these can be integrated into the vector design to increase the chances of successful and stable expression.

- Use non-retroviral vectors capable of carrying 100kb or more of transgenic sequence;
- Add "Insulators" to trangenes, i.e. DNA regions surrounding genes/gene clusters capable of preventing interaction with neighbouring genes. A good example would be 5'HS4 region from the locus control region (LCR) of chicken β–globin locus (Taboit-Dameron *et al.,* 1999).
- Use long genomic DNA fragments (100kb+) surrounding the gene of interest;
- Avoid silencer elements by constructing modified sequences where silencer elements are progressively deleted or mutated;
- Use site-directed integration by homologous recombination e.g. The Cre-Lox system.

Insulators may not only stimulate transgene expression by preventing local formation of inactive heterochromatin, but also actually act as chromatin openers that locally induce a hyperacetylation of histones and demethylation of DNA (Bonifer, 1999)

## Mosaicism

Mosaicism or 'variegated' expression of transgenes within a single organism is a common problem. This does not pose a serious problem in fish or mice where large numbers of transgenic animals can be produced and appropriate animals (i.e. those carrying the transgene in their germ line or reproductive cells) selected and subsequently bred. However mosaicism poses a major impediment for the application of transgenic technologies in livestock species where long generation intervals, low numbers of offspring and high costs of animal maintenance make breeding costly (Wall, 1997).

Mosaicism can arise in a number of ways.

1. Position-dependent inactivation of gene expression in a fraction of cells that generate a particular tissue. This "on or off" expression state is then maintained through future cell divisions
2. High transgene copy number and tandem head-to-tail arrangements make the DNA more prone to rearrangement, and as a result different cell subsets may be generated and may result in silencing of expression in a subset of cells.
3. Integration of the transgene into the chromosomes at different stages of cell division. Microinjection of the transgene vector into the pronucleus does not guarantee immediate integration. If integration occurs during the first cell cycle all cells would be expected to be transgenic. If integration doesn't occur until the second cell cycle, only 50% of cells would be transgenic, and for the third and fourth cell cycles the rate of transgenic cells would be 25 and 12.5% respectively, and so on. Integration before or after DNA replication at each cell cycle stage will also affect the efficiency of transgenesis (Chan *et al.,* 1999).

The risk of mosaicism can be reduced by the following methods.

1. Selective breeding from the mosaic founder animals to produce pure transgenic offspring. This approach is well demonstrated in the development of transgenic salmon expressing growth hormone under the control of the AFP promoter (Fletcher *et al*. 2003).
2. Screening to remove mosaic transgenic embryos before implantation into recipient females. The potential for screening has been demonstrated by Chan and colleagues (1999) using green fluorescent protein expression as a reporter of transgene expression. Embryos were examined by fluorescent microscopy; only transgenic blastomeres fluoresced. In the future, this may be a means of selecting transgenic, non-mosaic embryos.
3. Single-copy gene insertion by targeted insertion (homologous recombination) once this is economically feasible for animals other than mice (Bronson *et al.,* 1996).
4. Use of very long constructs (e.g. artificial chromosomes) to increase distance between repeat elements and therefore decrease risk of chromatin condensation.

5. Retroviral vectors can be introduced into the oocyte during metaphase II of the second meiosis and this has been demonstrated to result in a high probability of integration (Chan *et al.,* 1998). As a result, the genes are inserted before fertilisation so the resulting offspring should not be mosaic.

### Assessing gene stability for other purposes

While stability of expression is important for successful transgenic animal production, just as important is the question of "environmental stability", where this term implies:

- The likelihood of the gene spreading throughout a wild population following release or escape of the transgenic animal.
- The likelihood of the vector spreading to other animals including human via consumption.
- The likelihood of novel diseases being initiated by the process of transgenesis.

We are not in a position to put values on any of these likelihoods, but rather have chosen to list some of the researchable questions that might need to be answered. For example:

- How stable is the vector under a range of environmental conditions
- What is the likelihood of reactivation of the vector?
- What is the likelihood of vector retransposition?
- To what extent is the transgene in unrelated organisms?
- How stable is transgene to food processing?
- How stable is the transgene to digestion in the stomach or accidental introduction to an animal's bloodstream?

The molecular techniques discussed above have important applications to the assessment of environmental stability. While detailed discussion of environmental or health-associated impacts of transgenic animals is beyond the terms of reference of this review, we believe it is important to note that the study of environmental stability will be an important area of research in animal transgenics in the future. Responsibility for risk assessment and monitoring of transgenic animals falls within the jurisdiction of the OGTR in Australia and ERMA in New Zealand. For detailed review of the risks associated with transgenic animals and strategies for assessment and containment, please refer to the following reviews (Maclean, 2003; Sang, 2003; Maclean and Laight, 2000; Muir and Howard, 1999) as well as the OGTR (http://www.ogtr.gov.au/) and ERMA (http://www.ermanz.govt.nz/) web pages.

## The future

Since the production of the first genetically modified or transgenic animals in 1976, and the report of physiological effects of an introduced gene in mice in1982, the primary limitations to the advancement of the technology applied to livestock species, have been the inefficiency and expense involved. Consequently a great deal of research has focused on the background science and on techniques for improving the efficiency.

The isolation and culture of totipotent stem cells from livestock species will open up new possibilities for germline modification (Wheeler and Walters, 2001) and this advance seems inevitable. Further advances in sperm mediated transfer (Lavitrano *et al.,* 2002; Orwig *et al.,* 2002) will also open new possibilities for genetic modification including gene inactivation employing RNA interference (Rubinson *et al.,* 2003). Quantitative genetic and functional genomic studies, supported by the complete genome sequences of the production animals, will identify new targets for optimisation of production traits via transgenesis.

A second reason for slow commercialization of GM animals is the lack of capacity so far to incorporate novel functions from other species, in the way that companies have used herbicide resistance genes for the commercial launch and adoption of GM plants. So far, with a few exceptions,

animal researchers have simply attempted to change the level of existing functions, or attempted to produce pharmaceuticals and biomaterials.

## Recommendations and specific responses

### Description of how and why

a) An accurate description of transgenic techniques currently used or likely to be used in the next five years;

b) Which species, likely to enter the food supply for sale, have been used and what numbers might be involved (Table 5);

c) What traits are being transferred and expressed?

The responses to each of these questions make up the body of the text and associated tables.

### Evaluating the transgenic animals and food products

In this review, we were asked:

d) What the transgenic animals are being used for e.g. research, food production, fibre production, or to produce therapeutics;?

We have answered this generally in the text, and specifically in Tables 2 and 3.

e) How fitness/adequacy of animals is evaluated (i.e. what data is generated - phenotype/biochemical).

We recommend the approach taken by Adams *et al* in a series of recent publications describing growth hormone transgenic sheep. We conclude that this is a reasonable benchmark for thoroughness and transparency.

f) What foods are likely to be derived from transgenic animals or their progeny, both domestic and likely to be imported (penetration of by-products (e.g. meat, milk from animals engineered to produce biopharmaceuticals *etc*.) into the food supply), and associated compositional data.

We have provided information on animal product composition (Table 4) where it was freely available.

g) Is there any comparative information between the transgenic and the non-transgenic counterpart (e.g. compositional data of meat, milk etc).

Again, we have provided what data is freely available (Table 4), and conclude that collection and dissemination of data should be a priority for the developers of transgenic animals.

h) What methods are used to determine the stability of the transgene i.e. at the genotypic and phenotypic levels.

We have highlighted a number of processes that have been used to assess the stability of transgenes, though we have not approached the issues around assessment of risk in relation to instability of transgenes.

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# Appendices

**Appendix 1: Glossary of terms**

**AFP** (AntiFreeze Protein): peptides produced in the serum of some artic fish that lower its freezing point.

**alleles**: alternative forms of a gene which occupy the same position on a chromosome

**autonomous transposon**: small sections of DNA carrying a gene and other information, and capable of integrating with the genome

**biochemical**: pertaining to the measurement of chemicals within cells and tissues.

**biochemical expression profiles**: the broad range of chemicals that are produced by a cell via its genes, under certain conditions.

**Bioinformatics**: the computational and mathematical background to modern biology and genomics; bioinformaticians can be database specialists, statisticians or computer programmers

**cDNA:** a strand of DNA whose sequence is complementary to the a corresponding sequence of RNA

**cell line**: population of cells capable of dividing indefinitely in culture

**centromere**: a constricted region of a chromosome that includes the site of attachment to the mechanism that separates chromosomes during cell division – the spindle apparatus

**chimaera:** an animal that is a mixture of cells derived from two separate embryos from the same or different species

**chromosome:** a large DNA molecular chain in the cell along which genes are located

**conspecifics**: animals of the same species but different genotype at the locus of the transgene

**DEXA :** Dual Energy X-ray Absorptiometer

**DNA:** deoxyribonucleic acid, containing the genetic information that is passed from one generation to the next. It is a long, usually double-stranded molecule made up of the bases guanine, G; adenine, A; thymine, T and cytosine, C covalently linked to a sugar, deoxyribose, and to phosphate groups.

**enhancer:** a sequence of DNA that increases the use of promoter sequences. Together they control transcription and hence expression of genes

**enucleated:** a cell without a nucleus

**epigenic:** (epi = outside), caused by factors other than genetic

**epistasis**: interaction between nonallelic genes in which the presence of a certain allele at one site (locus) prevents expression of an allele at a different locus.

**expression:** not all genes are active. When a gene is read and the product of the gene (a protein) is produced, the gene is said to be expressed.

**FSANZ**: Food Standards Australia and New Zealand;

**gene**: the basic unit of heredity; an ordered sequence of nucleotide bases, comprising a segment of DNA. A gene may contain the sequence of DNA that encodes one protein chain. Each animal has two similar or dissimilar copies (alleles).

**genome**: the entire chromosomal genetic material of an organism

**genotype**: the genetic make-up of an organism

**genetically modified organism –GMO- (following the Royal Society):** means an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.

**germinal disc**: the entrance of the channel leading to the centre of the yolk. The germinal disc is barely noticeable as a slight depression on the surface of the yolk.

**GFP**: (green fluorescent protein) Used as a marker for successful transgenesis.

**GH**: growth hormone from various species.

**heterozygous:** having one or more pairs of dissimilar alleles on corresponding chromosomes, ie, the two alternative forms of a gene for a characteristic are different homozygous having identical rather than different alleles in corresponding positions on homologous chromosomes. The two alternative forms of a gene for a characteristic are the same and therefore the organism will breed true for that characteristic.

**IGF-1:** insulin-like Growth Factor 1 - a factor which can be measured in blood and has been shown to be associated with feed efficiency and fatness traits in pigs.

**intron/intronic**: a section of DNA that is transcribed, but is usually removed from within the mRNA transcript by splicing together the sequences (exons) on either side of it as a final step of the transcription process. In the past, it was generally considered to be a "nonfunctioning" portion of the DNA molecule. Also, sometimes a given intron remains in the transcript (e.g., via alternative splicing), resulting in a different protein expressed by the same gene.

**likelihood**: the probability of a particular outcome. We assume that a probability of 0.000001 is still worth noting and have not applied value judgements about levels of acceptably low likelihood.

**modern biotechnology:** the application of recombinant nucleic acid techniques

**mosaicism**: the state in which cells in a tissue have different genotypes but are derived from a single zygote.

**mutagenic:** a substance causing changes in DNA

**nucleus**: an organelle a (specialised structure) cell containing DNA

**nutriceutical**: a food or portion of food (e.g., a vitamin, essential amino acid, *etc*.) possessing medical or health benefits (to the organism that consumes that nutriceutical)

**peptide:** biologically important class of molecules that can exist separately or be part of a protein

**phenotype:** the appearance or other characteristics of an organism, resulting from the interaction of its genetic constitution with the environment

**plasmid**: An independent, stable, self-replicating piece of DNA in bacterial cells that is not a part of

the normal cell genome and that never becomes integrated into the host chromosome; may be linear or circular. Plasmids are commonly used in recombinant DNA experiments as acceptors of foreign DNA.

**pleiotrophy:** the multiple functions and phenotypic expressions of a single gene

**polygenic:** caused by many genes

**Polymerase-chain reaction (PCR)**: a polymerase (DNA replicating enzyme)-driven method allowing the targeted amplification of specific regions of DNA from a sample

**polymorphic**: Having many forms. In this context, it refers to multiple forms of a gene that lead to slight but measurable variation in a phenotype.

**promoter:** a region of DNA involved in binding the enzyme that reads the message on the DNA

**recessive condition**: a condition that requires two affected genes to be inherited, one from each parent, before causing a major change in the animal

**reporter gene**: one that, when altered, signals its presence under examination

**(m)RNA**: (messenger)ribonucleic acid: the string of nucleotides (usually single-stranded) that is "transcribed" from DNA

**RNAi**: RNA Interference**;** this term refers to what happens when short strands of (complementary) double-stranded RNA (dsRNA) are introduced into living cells. These effects can be utilized by scientists to cause gene silencing/knockout.

**smoltification**: suite of changes, including development of the silvery colour of adults and a tolerance for seawater, that takes place in salmonid parr as they prepare to migrate downstream and enter the sea

**somatic:** of the body

**stem cell**: one that has the capacity to renew itself as well as to produce more specialised progeny

**teleosts**: bony fish of the subclass Teleostei

**telomere**: assemblies consisting of protein and DNA sequences (that do not code for proteins), which are located at the ends of chromosomes

**teratogenic:** a substance that causes prenatal abnormalities

**totipotential:** a cell having the capability to form any cell (see stem cell)

**trait**: attribute or characteristic of animals that can be improved genetically (for example, growth rate, fertility, carcase or meat quality etc.)

***trans*-acting**: acting – having an effect - at a distance, as opposed to *cis*-acting : acting adjacently

**transcription:** the process of reading DNA

**transfection**: insertion of DNA segments (genes) into cells

**translation**: the process by which messenger RNA sequence directs the incorporation of amino acids into protein: occurs on a ribosome

**trochophore**: early, ciliated planktonic larval stage of an annelid or mollusc before segment proliferation begins

**xenotransplantation:** transplantation of tissues from one species to another

**Appendix 2: Terms of Reference of the Consultancy**

**FOOD STANDARDS AUSTRALIA NEW ZEALAND**
**TRANSGENIC ANIMAL PRODUCTION REVIEW**

**Proposed Terms of Reference**

Food Standards Australia New Zealand (FSANZ) regulates the safety and labeling of food produced using gene technology under Standard 1.5.2 of the Food Standards Code. Under this Standard**, a food produced using gene technology** means a food which has been derived or developed from an organism which has been modified by gene technology, and **gene technology** is defined as meaning "recombinant DNA techniques that alter the heritable genetic material of living cells or organisms".

FSANZ is seeking a review of the extent and use of transgenic animal production in both Australia and New Zealand. Food produced from animals that have been cloned, or have been developed using cell fusion techniques, are not captured under Standard 1.5.2 and are the subject of a separate review and will not be considered in the current exercise.

The focus of this review will be on what enters or is likely to enter the food supply for sale. The review will need to cover two broad categories:

**2) Description of how and why**

a) An accurate description of transgenic techniques currently used or likely to be used in the next five years;

b) Which species, likely to enter the food supply for sale, have been used and what numbers might be involved;

c) What traits are being transferred and expressed.

**3) Evaluating the transgenic animals and food products**

a) What the transgenic animals are being used for e.g. research, food production, fibre production, or to produce therapeutics;

b) How fitness/adequacy of animals is evaluated (i.e. what data is generated - phenotype/biochemical)

c) What foods are likely to be derived from transgenic animals or the progeny, both domestic and likely to be imported (penetration of by-products (e.g. meat, milk from animals engineered to produce biopharmaceuticals etc) into food supply), and associated compositional data;

d) Is there any comparative information between the transgenic and the non-transgenic counterpart (e.g. compositional data of meat, milk etc);

e) What methods are used to determine the stability of the transgene i.e. at the genotypic and phenotypic levels.

**Appendix 3: The review team**

The work has been completed by a team comprised of CSIRO scientists and a business services librarian, each contributing in areas of their expertise. The final report has been through CSIRO internal review, as well as a brief consultation with FSANZ to confirm the general breadth and specificity of the report.

The following people contributed directly to the review, and brought the stated expertise to the content.

Dr. Gregory Harper: biochemist with specialist knowledge of muscle food production systems; muscle developmental biology; team leader and principal author of the report.

Dr. Alan Brownlee: detailed knowledge of animal developmental biology and transgenesis.

Dr. Thomas Hall: specialist knowledge of fish developmental biology, aquaculture systems and molecular biological techniques.

Dr. Robert Seymour: molecular biologist with specialised knowledge of wool biology and the methodologies involved in transgenesis.

Dr. Russell Lyons: molecular biologist with detailed knowledge of aquaculture genetics and the methodologies involved in transgenesis.

Mr. Patrick Ledwith: business services librarian with expert knowledge of information systems and search strategies.

## Table 1: Transgenic terrestrial livestock species (existing or predictable developments)

| *Animal* | *Genes introduced or deleted* | *Performance criteria (for both directly affected and pleiotrophic traits)* | *References and notes* |
|---|---|---|---|
| | **Transgenic animals produced for food production applications** | | |
| Bovine | Various including recombinant antibodies; beta and kappa caseins; | Production of modified milk proteins | Houdebine, 1998; Pintado and Gutierrez-Adan, 1999; Bösze *et al.,* 2001; Houdebine 2002; Brophy *et al.,* 2003; Wall *et al.,* 1997; Zuelke, 1998; ERMA Application: GMD99110 |
| Bovine | Intestinal lactase | Reduction of lactose in the milk | Jost *et al.,* 1999; Whitelaw, 1999 |
| Bovine | Lysostaphin | Mastitis resistance | Houdebine 2002; Kerr *et al.,*2001; Wells, 2001. |
| Bovine | β-lactoglobulin | Increased production of this protein in milk, as well as increased growth and disease resistance in calves feeding on the milk. | Bremel *et al.,* 1996 and Bremel *et al.,*patent; 1999; |
| Bovine | Genes yet to be identified | Modified milk composition and reduced allergenicity in humans drinking the milk. | Maga and Murray, 1995 |
| Bovine | PrP | Reduced susceptibility of the cattle to Transmissible Spongiform Encephalopathies i.e. BSE | Denning *et al.,* 2001 |
| Bovine | Genes yet to be identified | Increased resistance to Trypanosomiasis (proposed) | Mattioli *et al*., 2000 |

| Animal | Genes introduced or deleted | Performance criteria (for both directly affected and pleiotrophic traits) | References and notes |
|---|---|---|---|
| Ovine | Growth hormone | Increased growth rates, increased feed conversion efficiency, decreased carcass fatness, and increased lactation.<br><br>The animals skeletal development, general health, resistance to internal parasites and reproductive performance were monitored over a 3.5 year period. More recently, muscle development in the context of meat eating quality traits are under investigation. | This has been a twenty year project. In total about 70 sheep have been in WA in recent times, though the growth hormone gene was inactive in about half. Some animals were also kept at CSIRO's laboratories at Prospect and Armidale. Animals were run under paddock conditions and inspected at least every 2 weeks for 3.5 years. Animals grew faster and were less fat, but had similar amounts of muscle. Their reproductive capacity was slightly impaired. Lactation was substantially enhanced, and the milk was not greatly different in composition. Detailed measurements were also carried out on hormonal functions under animal house conditions (Kadokawa *et al.* 2003a and 2003b). Work on the development of skeletal muscle is currently underway in collaboration between CSIRO and UWA. General references see Ward (2000). |
| Ovine | Genes from the synthetic pathway for sulphur-containing amino acids | Increased linear growth rate of the wool shaft, and perhaps improved follicle growth capacity. Increased muscle growth in animals at pasture would be expected. | Much of this work was inaccessible to the team, because of commercial in confidence arrangement. We were told that there were about 100 $G_0$, $F_1$ and $F_2$ animals. Key papers include Su *et al.,* (1998), Ward (2000), Bawden *et al.* (1995) and Bawden *et al.* (1999). |
| Ovine | IGF-1 with a keratin promoter | Increased wool growth | Su *et al*., 1998 |
| Ovine | Genes for specific keratins expressed in wool follicles | Altered wool properties, including increased lustre and strength | Powell *et al*. 1994 |

| Animal | Genes introduced or deleted | Performance criteria (for both directly affected and pleiotrophic traits) | References and notes |
|---|---|---|---|
| Ovine | Visna virus envelope | Reduced pathology due to *Maedi-Visna* virus, which causes encephalitis, pneumonia, and arthritis. | Clements *et al.,* 1994 |
| Ovine | PrP | Reduced susceptibility to Transmissible Spongiform Encephalopathies i.e. scrapie | Denning *et al.* 2001 |
| Ovine | Myostatin | Determine the extent of altered muscling characteristics. | ERMA application: GMD99052 |
| Caprine | Lysostaphin | Cure or prevention of *Staph. aureus* mastitis. | Fan *et al.,* 2002 |
| Porcine | Insulin-like growth factor 1 | Increased growth rate and reduced carcass fatness. Carcass composition has been confirmed by DEXA. There have been some anecdotal reports of altered skin tone; slightly shinier. | Nottle *et al.,* 1997; Pursell *et al.,*2001; Wheeler and Walters, 2001 |
| Porcine | Porcine growth hormone under metallathionine control | Increased growth rate and reduced carcass fatness. Improved feed conversion efficiency. | A number of labs including Adelaide University with Bresagen (Bresatec)[http://www.bresagen.com.au/rep_bio.asp]; USDA at Beltsville. |
| Porcine | Bovine α-lactalbumin | Increased growth rate in piglets; 10% increase in weight gain. Content and timing of the sows | Bleck *et al.,*patent; Bleck *et al.,*1998; and University of Illinois |

| Animal | Genes introduced or deleted | Performance criteria (for both directly affected and pleiotrophic traits) | References and notes |
|---|---|---|---|
| | | α-lactalbumin expression. Also investigated milk lactose concentration. | |
| Porcine | Monoclonal antibodies | Resistance to porcine gastroenteritis. | Saif and Wheeler, 1998; Sola *et al.,* 1998. |
| Porcine | Spinach Stearoyl CoA desaturase | Modified lipid composition (increased unsaturated fats) | Iritani 2002 |
| Porcine | *E. coli* Phytase expressed in saliva | Initiation of phytate utilisation by the pig, and hence a reduction in waste phosphorous. The authors state that the pigs have "similar health status to non-transgenic pigs", that "most grow at rates similar to non-transgenic pigs" and that "they appear to have similar reproductive characteristics". | (Golovan *et al.,*2001; Ward, 2001); The novel trait of the EnviropigTM enables it to degrade the indigestible phytate and absorb the phosphate eliminating the need to supplement the diet with readily available phosphate, and as a consequence the phosphorus content of the manure is reduced by as much as 75%. Digestion of the phytate also leads to improvements in digestion of minerals, proteins and starch in the diet. |
| Porcine | No genes specified | Increased disease resistance | Wheeler and Walters, 2001 |
| Porcine | No genes specified | Increased litter size (proposed) | Wheeler and Walters, 2001 |
| Galline (chicken) | *Leucosis* virus envelope | Increased disease resistance | Chen *et al.,* 1990; Crittenden and Salter, 1992. |
| Murine (as a model) | Lysostaphin expression in mammary glands | Potential to confer protection against *Staph* infection | Kerr *et al.,* 2001. |

| Animal | Genes introduced or deleted | Performance criteria (for both directly affected and pleiotrophic traits) | References and notes |
|---|---|---|---|
| Murine | Stearoyl-CoA desaturase | Modified milk fat composition; increase unsaturated fatty acid proportion. | Murray, 1999. |
| Caprine | Rat Stearoyl-CoA desaturase | Modified milk fat composition; increase unsaturated fatty acid proportion. | Murray, 2003<br>As yet unpublished but results were presented at the transgenic Animal Research Conference IV 2003, Lake Tahoe, CA |
| Caprine | Human lysozyme | Modified milk fat composition; enhanced immune response for animal and consumer, while inhibiting growth of harmful bacteria | Murray, 2003<br>As yet unpublished but results were presented at the transgenic Animal Research Conference IV 2003, Lake Tahoe, CA |
| Murine | *Clostridium thermocellum* endoglucanase E | Fibre digestion in monogastric production animals | Hall *et al.*, 1993. See also Ali *et al*. 1997; Zhang *et al.* 1999. |
| Murine | Isocitrate lyasae and malate synthase genes from the bacterium *E. coli* | Introduction of the glyoxylate cycle to a mammal to allow the net synthesis of glucose directly from acetate | Saini *et al*., 1996 |

| *Animal* | *Genes introduced or deleted* | *Performance criteria (for both directly affected and pleiotrophic traits)* | *References and notes* |
|---|---|---|---|
| **Examples of transgenic animals developed for pharmaceutical or biomaterial applications** | | | |
| Caprine | Human antithrombin III | Production in blood plasma, purification then application in the treatment of human antithrombin deficiency | Genzyme transgenics (Edmunds *et al.* 1998; Baguisi *et al.* 1999) |
| Caprine | Spider silk | Spider silk protein is expressed in the milk, and purified by classical techniques. | Nexia Biotechnologies Inc. has identified a strain of dwarf goats from West Africa that naturally Breeds Early and Lactates Early (BELE®), which reduces transgenic protein production time as compared with sheep, cows, and standard goats. For example, male BELE® goats are sexually mature as early as 15 weeks of age while standard male goats are sexually mature at 30 weeks of age. This allows a transgenic herd to be produced more quickly. The reduced time from lab quantities to production quantities of protein in turn allows an earlier start of clinical trials or product commercialization. http://www.nexiabiotech.com/. |
| Porcine | α (1,3) galactosyltransferase | Modification of cell surface antigens so as to minimise rejection of transplanted organs in humans | (Harrison *et al.* 2002; Ramsoondar *et al.* 2003) |
| Porcine | Human haemoglobin | Expression in pig blood, purification and used in blood substitutes | (Sharma *et al.* 1994; Rao *et al.* 1994; Logan and Martin 1994) |
| Bovine | Human antibody genes | Expression in the serum | (Robl *et al.* 2003) |

| *Animal* | *Genes introduced or deleted* | *Performance criteria (for both directly affected and pleiotrophic traits)* | *References and notes* |
|---|---|---|---|
| Ovine | Human α−1-antitrypsin | Expression in milk, application in the treatment of cystic fibrosis and emphysema | PPL Therapeutics (Carver *et al.* 1993). Could be as many as 2000 of these three transgenic sheep lines in NZ. ERMA application: GMF98001 |
| Ovine | Human tissue plasminogen activator | Expression in milk, application in treatment for blood clotting disorders | PPL Therapeutics |
| Ovine | Human Factor VIII | Expression in milk, application in treatment of blood clotting factor disorders | PPL (Niemann *et al.* 1999) |
| Murine | Growth hormones from various species | Production in urine suggesting the potential application of the bladder as a biofactory. | Kerr *et al.,* 1998. |
| Galline (chicken) | Bacterial Beta-lactamase | Hen as a bioreactor: production of exogenous protein in egg white | Harvey and Ivarie 2003. Progress reviewed in Ivarie 2003. |

## Table 2: Transgenic food fish/shellfish species, references and research groups

| Species | Country | Gene | Method | Reference |
|---|---|---|---|---|
| Common carp *Cyprinus carpio* | Israel | Chinook salmon gene, carp β-actin promoter AND carp β-actin promoter, carp growth hormone. | Microinjection of fertilized eggs | Moav *et al*., 1995, Hinits & Moav 1999. |
| | Canada | Chinook salmon growth hormone, ocean pout AFP promoter | Microinjection of fertilized eggs | Du *et al.,* 1992, Saunders *et al.,* 1998, Fletcher *et al.,* 1992, Cook *et al.,* 2000. |
| Yellow River carp *Cyprinus carpio* | China | Grass carp growth hormone, promotor unknown | Microinjection of fertilized eggs | Anecdotal information in the Pew Initiative report (2003), also http://www.bulletin.ac.cn/ACTION/2000102601.htm |
| Chinook salmon *Oncorhychus tshwytscha* | Canada and New Zealand | Chinook salmon growth hormone, ocean pout AFP promoter | Microinjection of fertilized eggs | Stevens & Devlin 2000. ERMA application: GMD01239 |
| Coho salmon *Oncorhychus kisutch* | Canada | Chinook salmon growth hormone, ocean pout AFP promoter | Microinjection of fertilized eggs | Stevens & Devlin 2000. |
| Cutthroat trout | Canada | Chinook salmon growth hormone, ocean pout AFP promoter | Microinjection of fertilized eggs | Devlin *et al.,* 1995b. |
| Rainbow trout *Oncorhynchus mykiss* | Canada | Chinook salmon growth hormone, ocean pout AFP promoter | Microinjection of fertilized eggs | Devlin *et al.,* 2001. |

| Species | Country | Gene | Method | Reference |
|---|---|---|---|---|
| | Finland | Sockeye salmon growth hormone | Microinjection of fertilized eggs | Pitakanen *et al.,* 1999. |
| Hornorum tilapia *Oreochromis hornorum* | Cuba | Tilapia growth hormone, CMV promoter | Microinjection of fertilized eggs | Hernandez *et al.,* 1997. Martinez et al., 1999, 2000. |
| Nile tilapia *Oreochromis niloticus* | Germany | Human growth hormone, mouse metallothionein-I promoter | Microinjection of fertilized eggs | Brem *et al.,* 1988. |
| | UK | Chinook salmon growth hormone, ocean pout AFP promoter | Microinjection of fertilized eggs | Rahman *et al.* 1998, Rahman & Maclean 1999, Rahman *et al.,* 2001. |
| Silver sea bream (*Sparus sarba*) | Taiwan/USA | Rainbow trout growth hormone, carp β-actin promoter | Electroporation of sperm/liposomal transformation of male gonads prior to mating | Lu *et al.,* 2002. |
| Arctic charr (*Salvelinus alpinus*) | Finland | Sockeye salmon growth hormone, sockeye salmon histone 3. Also Atlantic salmon GH. | Microinjection of fertilized eggs | Krasnov *et al.* 1999. Pitkanen *et al.* 1999. |
| Northen Pike (*Esox lucius)* | USA/Israel/Spain | Chinook salmon growth hormone, carp β-actin promoter | Microinjection of fertilized eggs | Gross *et al.* 1992. |

| Species | Country | Gene | Method | Reference |
|---|---|---|---|---|
| Black Porgy (*Acanthopagrus schlegeli*) | Taiwan/Japan | | | Tsai & Tseng 1994. |
| Ayu (*Plecoglossus altivelis*) | Taiwan | Rainbow trout growth hormone, carp β actin promoter | Electroporation of sperm | Cheng *et al.* 2002. |
| Mud Loach (*Misgurnua mizolepis*) | South Korea | Mud loach β-actin promoter, mud loach growth hormone | Microinjection of fertilized eggs | Nam *et al.,* 2001. |
| Japanese abalone (*Haliotis diversicolor suportexta*) | Taiwan | Chinook salmon growth hormone, ocean pout AFP promoter | Electroporation of sperm | Tsai *et al.,* 1997. |

## Table 3: Compositional data from transgenic livestock and aquaculture species

| *Organisation* | *Transgenic Animal* | *Transgene* | *Measurements Recorded* | *Results* | *References* |
|---|---|---|---|---|---|
| CSIRO | Sheep | Growth Hormone | Fat Depth | 1.62 mm (trans.) v 1.95 mm (cont.) at 5 mo of age;<br>3.3 mm (trans.) v 4.9 mm (cont.) at 18 mo of age in Merinos | Adams *et al*., 2002 |
| | | | Eye Muscle Depth | 18.8 mm (trans.) v 19.7mm (cont.) at 5 mo of age;<br>21.8 mm (trans.) v 25.2 mm (cont.) at 18 mo of age in Merinos | |
| | | | Wool yield | 1.51 kg cfw (trans.) v 1.47 kg (cont.) cfw at 6 mo of age;<br>3.17 kg cfw (trans.) v 2.83 kg cfw (cont.) at 18 mo of age in Merinos | |
| | | | Liveweight | 62.9 kg (trans.) v 51.2 kg (cont.) in Merinos;<br>61.1 kg (trans.) v 57.0 kg (cont) in Poll Dorset cross. | |
| Agresearch NZ | Cow | β and κ casein | Fat | Within normal range for bovine milk. | Brophy *et al*., 2003 |
| | | | Lactose | Within normal range for bovine milk. | |
| | | | Minerals | Within normal range for bovine milk. | |
| | | | β casein | 18.3 mg/ml (20% above controls) | |
| | | | κ casein | 8.4 – 14.1 mg/ml (double the control) | |

| ***Organisation*** | ***Transgenic Animal*** | ***Transgene*** | ***Measurements Recorded*** | ***Results*** | ***References*** |
|---|---|---|---|---|---|
| | | | Total milk protein | Slightly increased | |
| USDA | Pig | Growth Hormone - somatotropin | Lipid composition and cholesterol content for three different gene constructs | 70-87% saturated fatty acids cf. controls;<br>60-84% total fat cf. controls;<br>69-89% monounsat. FA's cf. controls;<br>36-71% PUFAs cf. controls;<br>Loin eye area normal;<br>52-67% intramuscular fat cf. controls;<br>Cholesterol content normal rang;e<br>Tenderness (shear test) not different from normal. | Solomon *et al*. 1997 |
| BresaGen | Pig | Growth Hormone | Feed conversion<br>P2 fat depth<br>Muscle depth<br>IGF-1 | Ratios lower cf. littermate controls<br>Lower fat depth than controls<br>Normal range<br>Up to 44% higher circulating after zinc induction | Nottle *et al*. 1999 |

| Organisation | Transgenic Animal | Transgene | Measurements Recorded | Results | References |
|---|---|---|---|---|---|
| University of Illionois | Pig | Bovine Alpha-lactalbumin | Milk lactalbumin<br><br>Milk protein and total solids<br>Lactose concentration<br>Milk production | 50% increase in total throughout lactation; ration of bovine to pig lact.not constant.<br>No consistent significant differences over entire lactation.<br>Trend for higher lactose in transgenic pigs but only significant at day 0 of lactation.<br>Transgenics produce more milk (5.2 – 7.4 kg/day vs. 4.3 –6.7kg/day over first 9 days lactation) | Wheeler *et al*. 2001 |
| Aquabounty Farms, Canada | Atlantic salmon *Salmo salar* | Ocean pout AFP promoter, chinook salmon growth hormone CDS | Feed digestability, feed conversion, carcass composition (protein, ash, lipid, dry matter energy). | 10% increase in gross feed conversion efficiency. Increased moisture content, decreased protein ash, lipid, dry matter and energy content over the study period. | Cook *et al*., 2000 |
| University of Guelph, Canada | Atlantic salmon *Salmo salar* | Ocean pout AFP promoter, chinook salmon growth hormone CDS | Morphological characteristics of the intestine and pyloric caeca. | Intestinal surface area 1.5 times larger than control animals. Pyloric caeca surface area 1.2 times larger. | Stevens *et al*., 1999 |
| University of Guelph, Canada | Coho salmon *Oncorhynchus kisutch* | Pacific salmon metallothionein promoter/histone 3 promoter and growth hormone CDS | Morphological characteristics of the intestine. | Total intestinal surface area 2.2 times larger than in controls. | Stevens & Devlin 2000 |

| Organisation | Transgenic Animal | Transgene | Measurements Recorded | Results | References |
|---|---|---|---|---|---|
| University of Southampton, UK | Nile tilapia *Oreochromis niloticus* | Ocean pout AFP promoter, chinook salmon growth hormone CDS | Head length, total length, viscera mass liver mass, gonad mass (mixed rearing conditions) | 2.4% increase in head to total length index<br>5.9% increase in visceral-somatic index<br>14.7% increase in hepato-somatic index<br>39.1% increase in gonado-somatic index (male)<br>14.7% decrease in gonado-somatic index (female) | Rahman *et al*., 2001 |
| Chinese Academy of Sciences, China | Common carp (*Cyprinus carpio*) | Human growth hormone CDS, mouse metallothionein promoter | Whole body amino-acid profile. | Increased relative body proportion of lysine | Fu *et al*., 2000 |
| Auburn University, USA | Common carp (*Cyprinus carpio*) | RSV promoter, rainbow trout growth hormone CDS | Protein, fat, moisture content, amino-acid profile, fatty acid profile | Muscle protein content 1.35% higher. Fat content 0.49% lower, moisture content 4.87% lower. Observed values for aspartic acid, cystine, glutamic acid, histidine, lysine and threonine significantly higher. | Chatakondi *et al*., 1995 |
| University of Maryland, USA | Common carp (*Cyprinus carpio*) | RSV promoter, rainbow trout growth hormone CDS | Detailed morphometric measurements, dressing percentage. | Many changes in morphological measurements resulting in up to 5% increase in dressing percentage depending on transgenic strain. | Dunham *et al*., 2002 |
| University of Kuopio, Finland | Arctic charr *Salvelinus alpinus* | Sockeye salmon growth hormone, CMV promoter | White muscle fibre number, nuclear density | Increased muscle growth was associated with increased white muscle fibre number (i.e. hypertrophy), and increased nuclear density | Pitkanen *et al*., 2001 |

| ***Organisation*** | ***Transgenic Animal*** | ***Transgene*** | ***Measurements Recorded*** | ***Results*** | ***References*** |
|---|---|---|---|---|---|
| University of Kuopio, Finland | Arctic charr *Salvelinus alpinus* | Sockeye salmon growth hormone, CMV promoter | Muscle composition (biochemistry), rates of gas exchange | Decreased plasma triglyceride and cholesterol. | Krasnov *et al*., 1999 |

## Table 4: Numbers of transgenic animals in Australia and New Zealand

| *Organisation* | *Species* | *Transgene or target phenotype* | *Current numbers* | *Comments and other information* |
|---|---|---|---|---|
| Celentis Ltd, NZ, AgResearch | Bovine | bovine beta and kappa casein<br><br>and human myelin basic protein | 64 animals in total, made up of 32 founder animals and 32 G1 progeny some of which have been produced through IVF | No carcass composition data has been collected as yet, but animals could be made available to do so, subject to New Zealand ERMA approval. Normal lactation milk from the founder beta and kappa casein animals is currently being collected for compositional analysis. |
| CSIRO, Vertebrate Pest CRC partners | Carp and invertebrates | Sterility via disruption of early stage embryo-genesis, under the repressible control of an externally applied trigger molecule | Several dozen, transient expressors | The project ended in 2001. Given the early embryonic target genes, no later life effects on body composition were expected. |
| PPL | Sheep | Alpha-1 Antitrypsin | Until recently, 4000 sheep in New Zealand, of which 3000 are transgenic | Media releases from July 2003 indicate that partner Bayer has decided to suspend the project for 3 years. All animals may be culled. As of Sept. 2003 there is no indication that this has occurred. Prior to culling these sheep Environment Risk Management Authority (ERMA) must be notified. |
| SARDI | Sheep | Transgenics for novel wools traits | undisclosed | |
| CSIRO and collaborators | Pacific oyster (*Crassostrea gigas*) | repressible sterility | ~20,000 larvae only | |

| ***Organisation*** | ***Species*** | ***Transgene or target phenotype*** | ***Current numbers*** | ***Comments and other information*** |
|---|---|---|---|---|
| Austin Research Institute and XenoTrans Ltd, Victoria | Pig | Production of multiple transgenic pigs for future xenotransplantation | 30 pigs transgenic for different fluorescent protein genes<br>4 sows pregnant with transgenic litter as of July 2003 | |
| Dairy CRC | Bovine | Alpha S1 Casein for increased protein content in milk | 14 transgenic calves born but only 1 surviving | Further information regarding this research can be obtained from the "Cloning and other Genetic Advances" Fact sheet available at http://www.dairycrc.com/framenews.htm |

## Table 5: Methods used in creation of transgenic animals

| *Method* | *Description* | *Recent References* |
|---|---|---|
| Nuclear Transfer | Transfer of a nucleus from primary cells, immortal cells or transformed cells into a single cell embryo that as had its nucleus removed. Transfer of whole cells into enucleated embryos has been achieved in the pig. | (Zakhartchenko *et al.* 2001; Nagano *et al.* 2001; Prather *et al.* 1999; Niemann and Kues 2000; Forsberg *et al.* 2002; Aso *et al.* 2002; Leno and Forsberg 2002; Yang and Kubota ; Colman *et al.* ) |
| Homologous recombination | This process is primarily used to delete a gene from an animal via homology with known gene sequences. Commonly used to generate deletion mutants in mice. | (Behboodi *et al.* 2001; Rieth *et al.* 2000; Rieth *et al.* 1999; Rieth *et al.* 2000; Dymecki 2002; Zarling and Sena 2002) |
| Retroviral mediated transgenesis | This method is one of the most commonly used methods for the generation of transformed cells and is now more commonly used than pronuclear microinjection. | (Langford *et al 2001.* ; Fleury *et al.* 2003; Rubinson *et al.* 2003; Kubo and Mitani 2003; Pfeifer *et al.* 2002; Hamra *et al.* ; Wells *et al.* 1999; Baltimore *et al.* 2003; Ivarie 2003) |
| Marker technologies | Markers are required to track and select transgenic cells or embryos. The most commonly used marker is the green fluorescent protein (GFP). | (Matsui *et al.* ; Devgan and Seshagiri 2003; Bordignon *et al.* 2003; Richards *et al.* 2003; Prather *et al.* 2003; Lai *et al.* 2002; Chan *et al.* 2002; Park *et al.* 2001; Roh *et al.* 2000; Wan *et al.* ) |
| Sperm-mediated gene transfer | The sperm from a donor animal are transformed through the use of a cellular gene transfer technology (electroporation etc). The viable sperm is then used to fertilise a donor egg. | (Szczygiel *et al.* 2002) (Celebi *et al.* 2003; De Miguel and Donovan 2003; Lavitrano *et al.* 2003; Orwig *et al.* 2002; Feng *et al.* 2002; Brinster 2002; Chang *et al.* 2002; Kanatsu-Shinohara *et al.* 2002; Sato *et al.* 2002) |
| Microinjection | Microinjection has been the preferred method over the last 15 years. Is highly inefficient and being superseded by other methods. | (Langford *et al.* ; Baldassarre *et al.* 2003; Bagis *et al.* 2002; Bagis *et al.* 2002; Arat *et al.* 2001; Wall 1996) |

| ***Method*** | ***Description*** | ***Recent References*** |
|---|---|---|
| Transposon-mediated gene transfer | A method commonly used in drosophila now finding application in mammals due to the generation of new vectors. | (Dupuy *et al.* ; Sasakura *et al.* 2003; Harris *et al.* 2002; Horie *et al.* 2001; Fischer *et al.* 2001; Izsvak *et al.* 2000; Ivics *et al.* 1999) |
| Artificial chromosomes | Large independently relocating chromosomes that can be used to transport large fragments of DNA. These exist episomally in the cell of a transgenic animal. | (Robl *et al.* 2003; Poggiali *et al.* 2002; Gama *et al.* 2002; Kuroiwa *et al.* 2002; Nistala and Sigmund 2002; Casanova *et al.* 2002) |

## Table 6: Relative sensitivities of various detection techniques

| *Method* | *Target* | *Limit of Detection (Number of molecules)* |
|---|---|---|
| PCR | | |
| (one-step) | DNA | 100+ |
| (two-step nested) | DNA | Approaching 1 (most sensitive by far) |
| RT-PCR | RNA | 100+ (some loss of sensitivity [10-100 fold] due to inefficiencies related to cDNA synthesis) |
| Q-PCR (Real-time) | DNA | 10+ (it is possible to detect less but the likelihood of spurious non-specific product makes it dangerous to quantify below this number – therefore 10 is a safe cut-off) |
| | RNA | 100+ (some loss of sensitivity [10-100 fold] due to inefficiencies related to cDNA synthesis) |
| Northern Hybridisation | RNA | 10 000+ |
| Southern Blot | DNA | 10 000+ |