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Lowering Sperm Dose Rates in Frozen Semen for Bovine Artificial Breeding



THE UNIVERSITY OF
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By

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Abstract

The New Zealand Dairy industry relies on artificial breeding to produce high genetic merit replacement stock. Proven bull semen is extended and preserved as either ambient temperature product or as frozen doses. High fertility is essential to maintain synchronicity of lactation with the spring surge of grass growth.

To improve efficiency in the utilization of the very best bulls producers try to lower sperm dose rates without compromising fertility. Livestock Improvement Corporation's (LIC) Long Last Liquid™ (LLL) is most commonly used during the peak season and is their most important product. However, shortfalls and specifically targeted matings are met with frozen semen. Lowering sperm dose rates in both liquid and frozen semen maximises the usage of elite sires increasing genetic gain, lowering overheads and garnering premium prices for the semen producer.

A product for improving frozen semen technology was developed whereby a discrete quantity of pretreated semen was placed alongside a beneficial post-thawing redilution medium in a standard semen straw. This emulated a larger-scale process developed to freeze semen in times of low demand and redilution into the LLL form for use as an ambient temperature product. This rediluted product has been proved to enhance semen survival and to allow fertility to be maintained at sperm dose rates lower than the widely accepted standard for frozen semen.

The physical packaging of the semen into the industry standard single dose straw in a configuration that separated incompatible components was novel. This configuration was essential to prevent damage to the sperm during the freezing process from low solubility components in the post-thaw redilution medium. Separation between the semen and diluent within the straw was achieved by the introduction of a gas partition or air bubble between the two liquids.

A large-scale field trial showed that the new product could produce savings of up to 25% of the sperm needed for a semen dose to achieve equivalent fertility. Furthermore, the production cost per dose was lowered in comparison to the standard semen processing system used at LIC.

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Glossary of terms used in this thesis

AB. Artificial Breeding.

AI. Artificial Insemination.

BST. Bovine Somatotrophin hormone used to extend lactation in dairy animals.

BW. Breeding Worth. A measure of the genetic potential of an animal.

Caprogen. The liquid semen diluent used by LIC for its LLL products.**CASA.** Computer Aided Semen Assessment. Using a computer to analyse various morphometric and motility parameters of sperm to provide a picture of the viability of an ejaculate.

CASA Computer Aided Semen Assessment. Systems using computerized image analysis to quantify sperm motility and morphology parameters.

Cold-shock. A term describing the changes that occur when sperm are exposed to low temperature.

Cryogenic. Extremely low temperature.

Cryoprotectant. A substance that protects cells from freeze damage.

NZ. New Zealand.

CIDR-B. Controlled Intravaginal Drug Releasing device. Device to assist in oestrus synchrony in ruminants by emulating reproductive hormone release cycle.

Cryovet. A rival company to IMV that produces straws for frozen semen.

DataMate. Hand-held data logger used by LIC inseminating technicians to record information relevant to matings.

Flow cytometry. A system that analyses individual cell parameters using specific wavelengths of light to induce fluorescence of dyed cellular components.

Fonterra. New Zealand's largest dairy cooperative. Processes and markets over 90% of New Zealand's dairy production.

FPD. Farm Production Division of the NZ Dairy Board. A division of the NZ Dairy Board that was the forerunner of LIC and became part of LIC when it was incorporated.

IMV. Instrumentes du Medecine Veterinaire. A French company that developed the frozen semen technology used through most of the developed world.

Kaptan. Plastic used for cryogenic packaging.

KiwCross™. A breed of dairy cattle developed in New Zealand from Holstein-Friesian and Jersey ancestry.

KNEX. The code name given to the project and the frozen semen product described in this thesis.

LIC. Livestock Improvement Corporation. A large dairy breeding and milk testing company based in Hamilton, New Zealand.

LLL. Long Last Liquid™. Livestock Improvement Corporation's flagship ambient temperature semen product.

LN₂. Liquid Nitrogen. Used as cryogenic refrigerant.

Minitub. Minitub GmbH is the German company supplying PVC straws for LIC's LLL product.

mwco. Molecular Weight Cut-off. Term used to describe the cut-off point above which a dialysis membrane excludes molecules in solution.

NRR. Non-Return Rate. A measure of fertility derived from the ratio of females returning to service, that is, failing to conceive to a service.

NZDB. New Zealand Dairy Board. Former New Zealand government body established to oversee dairy production and marketing.

OSH. Occupational Health and Safety act that governs workplace safety.

PD. Pregnancy diagnosis. Most commonly performed by rectal palpation and/or ultrasound imagery.

PG. Prostaglandin. Hormone used in oestrus synchrony programs.

Pistolette. Inseminating device to contain the straw for deposition of semen into the female.

PVA. Poly Vinyl Acetate. Water soluble glue that can be powdered in its anhydrous state.

PVC. Poly Vinyl Chloride. Plastic used for many applications including straws for artificial breeding.

RDF. Rediluted Frozen Semen. An artificial breeding product used in NZ consisting of straws or bags of concentrated frozen semen that was thawed and rediluted in the semen production laboratory into a volume of Caprogen ready for use as liquid semen.

ROS. Reactive Oxygen Species. A term to describe the various oxidizing compounds that are formed during metabolism or in cellular breakdown.

SCC. Somatic Cell Count. An indicator of the level of infection in a milk sample. Used as a test for mastitis in lactating dairy animals.

Sempak. An artificial breeding product consisting of a straw of concentrated frozen semen that was thawed and rediluted in the field ready for use as liquid semen.

SPD. Sperm per inseminate Dose

SPS. Sire Proving Scheme. Program to breed and prove the genetic worth of sires.

TMR. Total Mixed Rations. Feed for intensively farmed cattle in feedlot systems.

Tris. Trishydroxymethylamine. An organic buffer used extensively as a component in frozen semen diluent.

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1 Introduction

The New Zealand Dairy industry is one of the most cost efficient producers of milk and processed dairy products in the world. It relies on pasture-based farming systems to produce cheap feed for animals bred to efficiently convert grass to milk products [2], [3; 4]. The seasonal surge in grass growth that occurs in spring is the most cost-efficient feedstock for the lactating cows. Cows have to be at the optimum point in their lactation to fully utilise the available feed. Therefore, parturition and hence, conception must occur to a strict timetable. High fertility is essential if the herd is to lactate at the right point in the calendar [5], [6], [7].

New Zealand farmers formed cooperatives such as Livestock Improvement Corporation (LIC) for provision of farm services such as animal evaluation, product testing and breeding of suitable dairy animals[8]. Semen research at LIC is mainly focussed on lowering sperm dose rates and extending the life of its liquid and frozen semen products. This maximises the usage of elite bulls proven to enhance production traits in their daughters. An over-riding consideration is that fertility must be maintained.

LIC's operation is based in two important products: Long Last Liquid™ semen (LLL), which is used during peak demand and deep frozen semen to supplement LLL or for use during off peak season.

Most frozen semen systems are constrained by high sperm dose rates compared to LLL, but LIC has previously developed a frozen semen system where semen frozen at high concentrations is rediluted into the LLL form for distribution. This product was named Rediluted Deep Freeze (RDF). RDF sperm dose rates are more favourable than other frozen systems. The major limitation of this product is its perishability after thawing and redilution. Its positive attribute of lowering sperm dose rates lead to this project.

There is a basic need therefore, for a single dose product capable of overcoming some of the deficiencies in LIC's existing products.

In this thesis, the product development is described along the principles of classical chemical product development [9].

That is:

- ◆ Identification of industry needs.
- ◆ Generation of ideas.
- ◆ Screening between ideas and selecting the most promising idea.
- ◆ Product manufacture.

The work is based on research located at LIC's semen research facility at Newstead, Hamilton, New Zealand and this thesis is limited to cattle artificial breeding. Furthermore, possible solutions are constrained by current practices, especially within the infrastructure developed for storing and distributing frozen semen.

2 The role of Artificial Breeding in the New Zealand Dairy Industry

2.1 Historical Perspective

The introduction of artificial breeding into the New Zealand dairy industry in the 1950's has made a major contribution to the genetic progress of the New Zealand dairy cattle population, which has resulted in increases in milk and milk component production. Production increases have also been garnered from improvements in management and feeding practices. However, commensurate with better pasture utilisation has been the improvement in feed conversion into milk products by cows selectively bred to maintain body condition and overall health while efficiently lactating, conceiving and maintaining pregnancy [10].

2.2 Livestock Improvement Corporation

Livestock Improvement Corporation (LIC) is a user owned company that was divested from the Farm Production Division (FPD) of the New Zealand Dairy Board (NZDB) when it was amalgamated with the two largest dairy companies in New Zealand to create the Fonterra Corporation. LIC services more than 75% of the dairy artificial breeding market with its flagship semen product 'Long Last Liquid' (LLL) TM semen. LIC administers a large sire-proving scheme and also manages the major milk testing service in New Zealand. LIC also markets advice, software and farm automation services to farmers and exports semen internationally [11].

The dairy cattle artificial breeding industry in New Zealand is severely constrained by the extreme seasonality of demand for breeding services. The majority of dairy farms schedule calving to allow the lactational peak to coincide with the spring grass growth surge to allow the most efficient utilization of available feed for conversion to milk [8].

Approximately 3.5 million calves of dairy or mixed dairy/beef breeds are born during the period mid-July to late August. Lactating cows are

required to return to oestrus within six to eight weeks of parturition and 65% to 70% of these animals are expected to conceive to their first insemination. This pattern maintains a 360-day calving interval, which is essential if cows are to calve at the appropriate time in the following season to maintain synchrony with the grass growth cycle.

LIC annually produces over three million doses of liquid and frozen semen from its highest genetic merit bulls. Only thirty bulls are used to perform these inseminations. The top ten bulls perform eighty five percent of the total inseminations. The remainder are used to make up shortfalls and to add diversity to the cattle population. The majority of inseminations are performed from mid-September to late November (see Figure 2.1). The limited life of the LLL product dictates that the logistics of such an operation are complex and well integrated. Semen produced at LIC's base in Hamilton has to be distributed to farms in all parts of the country over the subsequent 24 hours for use over the ensuing three days.

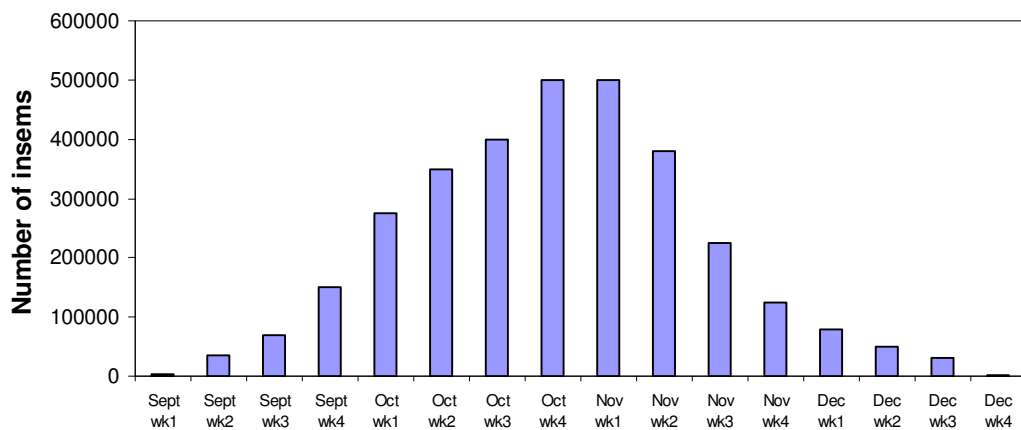


Figure 2.1: Weekly demand for inseminations in spring in New Zealand

Daily demand for inseminations is between 60,000 and 90,000 doses from the major dairy breeds (Holstein-Friesian, Jersey and Kiwicross™). Within each breed daily demand exceeds the ability of the top sires to produce semen in sufficient quantities. LIC then utilizes sires of lower genetic merit in LLL semen or uses its back-up system of frozen semen to

make up the shortfall. In practice, a combination of both products is used to enable LIC to guarantee that a minimum Breeding Worth (BW) average is delivered to each herd.

2.3 Liquid Semen Technology

The rapid dissemination of superior genetics throughout the New Zealand dairy population has been possible by the introduction of ambient temperature, low sperm dose rate semen diluents. The abundant supply of inexpensive semen from high genetic merit bulls is largely attributable to the efforts of Shannon et al; based at the Livestock Improvement Corporation semen research laboratory at Newstead, near Hamilton. They developed Caprogen diluent as a vehicle for ambient temperature semen preservation [12], [13].

Caprogen bestows the following protective effects on sperm at ambient temperature (6-26°C):

- ◆ Osmolarity is optimised for sperm survival [12], [14], [15].
- ◆ pH is controlled by the buffering effects of the medium [16], [17].
- ◆ Membrane stabilizers keep membranes fluid yet stable over the storage temperature range [18], [19].
- ◆ Further membrane stability and thermal protection is supplied by the addition of hen's egg yolk [20], [21], [22].
- ◆ Anti-oxidants control the effects of hydrogen peroxide and other reactive oxygen species [23], [24], [25], [26], [27], [28].
- ◆ Oxygen metabolism is reduced by reducing oxygen tension in the medium [29], [30], [31], [32], [33], [34].
- ◆ Metabolic nutrients are supplied as glucose [35], [36], [16].
- ◆ Antibiotics control bacterial growth in the medium and semen-borne pathogens [37], [38].

Packaged as a single inseminate dose in 65mm PVC straws sourced from Minitub GmbH in Germany, diluted semen is marketed as ‘Long Last Liquid’™ (LLL) [18], [19]. This system permits semen dose rates as low as 1×10^6 sperm per dose (SPD), in contrast to the common industry standard of 15 to 20 $\times 10^6$ SPD in frozen semen systems. LIC regularly achieves in excess of 250,000 inseminations per year per bull from its Holstein-Friesian sires. This compares favourably with international production of less than 60,000 inseminations per bull using frozen semen.

The use of liquid semen at sperm dose rates one-twelfth that of the frozen product has increased the rate of genetic gain in New Zealand dairy cattle [39]. Figure 2.2 shows the comparison between the averaged yearly production capabilities of Friesian and Jersey bulls when using the two semen extension technologies.

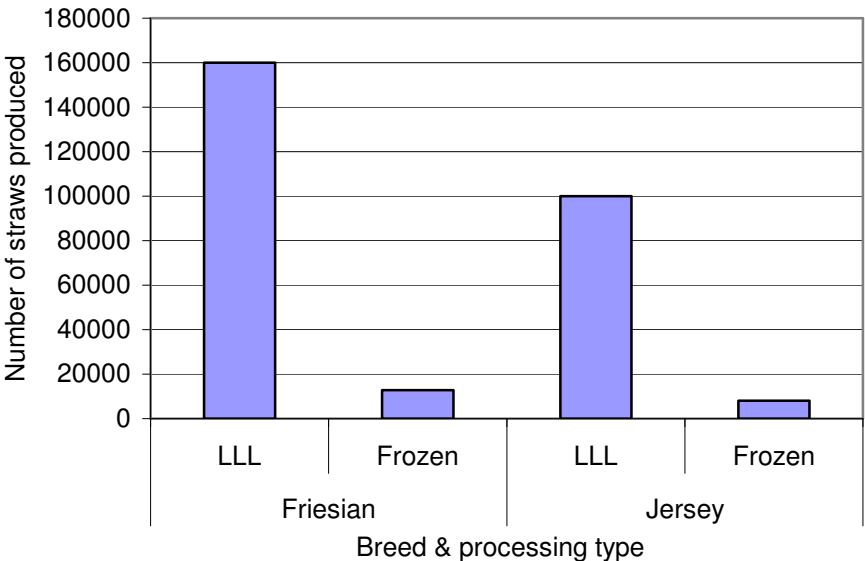


Figure 2.2: Average yearly production of straws using Long Last Liquid or frozen semen technologies for Friesian and Jersey Bulls

Semen is distributed in liquid form in insulated boxes at the local ambient temperature. Fertility of the semen drops measurably after four days of life and it must be replaced with a fresh batch. As the semen demand varies daily there can be either wastage or shortfall. Wastage is minimised

at times of peak demand during the period mid-October to late November as the demand almost invariably exceeds supply so all semen despatched is used up. Inevitably, despite the best efforts, the wastage or shortfall represents an added cost to the New Zealand dairy farmer both in terms of the price of semen and in lost genetic potential of unused inseminations from top sires.

2.4 LIC's Product Mix

Semen produced outside the spring mating period must be frozen as spring is the only time there is sufficient demand to trigger cost-effective LLL production. Distribution and utilization practices have to balance the usage of liquid and frozen semen delivery systems resulting in up to 10% of all inseminations being carried out with frozen semen [40].

The demand for high BW semen has lead LIC to foster an active Research and Development program to enhance both liquid and frozen semen technologies. Research is focused around two key drivers:

- ◆ lowered sperm dose rates for both liquid and frozen products which increases the usage of the highest rated bulls.
- ◆ increased lifespan for the liquid product which lowers logistics costs and delivers better genetics through more efficient utilization of the top bulls' output.

2.5 Frozen Semen Technologies

Dairy artificial breeding technology in the first world dairying nations almost invariably utilizes frozen semen. The single dose PVC straw developed by the Instrumentes du Medecine Veterinaire (IMV) company of l'Aigle, Normandy, France is the industry standard frozen semen package [41]. The IMV straw offers many attributes that forms a cost-effective package for artificial breeding companies and the farmers using their services:

- ◆ Compact dimensions. The IMV straw is 135mm long x 2.0 mm in diameter, nominally containing 250ul of diluted semen. As storage space under liquid nitrogen is a major cost to AB companies, the small dimensions of the straw allow a low unit cost per dose stored.
- ◆ High surface area to volume ratio. The rapid transfer of heat into and out of the straw during freezing and thawing minimises damage to sperm by shortening the exposure time at critical phase transition points.
- ◆ Convenient packaging. The straw serves as the storage, transport and delivery vehicle for the semen. It maintains physical separation from contamination and liquid nitrogen ingress, it is easily transportable and once thawed it becomes the cylinder/piston through which the semen is expelled into the cow.
- ◆ Ease of identification. Straws are easily labeled and are legible under cryogenic conditions. This is essential for accuracy of stock control.
- ◆ Inexpensive. At four cents each the straw has a low unit cost.

The above series of advantages have lead to widespread uptake of the IMV straw and its imitators. Of the 244 million doses of semen produced world wide each year for cattle AI, 232 million are frozen and this has lead to an industry infrastructure around this technology. This includes:

- ◆ Inseminating devices and techniques. A specialized inseminating gun (or Pistolette) has been developed for ease of loading and insemination.
- ◆ Cryogenic systems. Techniques for handling these straws include specialized plastic goblets to contain discrete numbers of straws, cryogenic vessels built specifically around the straw dimensions, straw counters, specific nomenclature and a variety of other infrastructural systems.

- ◆ Straw filling and handling equipment. Dedicated machinery has been developed to fill and seal these straws in batch lots. Commonly, AB laboratories process up to 20,000 straws daily. Controlled temperature-rate cryogenic freezers have also been specifically developed for IMV straws.

2.6 Frozen Semen Physiology

Polge's discovery of the cryoprotective effects of glycerol allowed the preservation of sperm indefinitely while frozen [42]. Optimisation of cryoprotectant level, osmotic balance, buffering, and the additional protective effects of egg yolk all contributed to the development of frozen semen diluents that allow inseminate rates as low as 12.5×10^6 SPD [43], [44; 45], [46], [1].

Diluent systems are based on isotonic buffering systems such as Trishydroxymethylamine (Tris)/citric acid or sodium citrate/citric acid [38]. These mitigate the build up of metabolic by-products such as lactic acid and maintain a near neutral pH. Diluents can also be based on naturally buffered compounds such as skim milk or coconut milk systems that include proteins that compete with toxic seminal plasma proteins for sperm membrane binding sites [20].

Egg yolk is used as a cryoprotectant membrane stabiliser in most frozen semen diluents. Egg yolk binds to sperm membranes and competes with seminal plasma proteins shown to be deleterious to sperm [22; 47], [48]. Water-soluble egg yolk lipoproteins have been shown to have protective effects by various researchers [22], [49], [50], [51], [52].

Other researchers have attempted to replace egg yolk proteins with soybean lecithins, as many first world countries cannot guarantee bacteria-free egg supplies [53], [54], [55].

Semen diluents also contain antibiotics to prevent bacterial growth in the media and to prevent disease transmission from organisms such as leptospira. Antibiotics are toxic to sperm and levels are adjusted to

maintain bacteriostasis rather than a complete kill which would also overwhelm the sperm. Raw ejaculated semen always carries an inoculum of bacteria and therefore semen processors aim to control growth while minimising toxicity to sperm [37], [38].

The main cryoprotectant ingredient in all frozen semen systems is the low molecular weight, sugar alcohol glycerol. The partitioning of salt solutions under freezing conditions and the consequent rise in osmolarity of the unfrozen supercooled portion leads to extensive membrane and cellular damage. Glycerol changes the dynamics of the formation of the interfaces between the areas of high and low osmolarity, minimising crystal formation and mitigating cellular damage. Glycerol, like many cryoprotectants, is toxic at the wrong concentrations. It can also induce osmotic shock if it is not introduced to the sperm cells progressively.

2.7 Rediluted Deep Frozen Semen (RDF)

Shannon and his co-workers also developed Rediluted Deep Freeze (RDF). This was a system for freezing semen at concentrations between 200 and 600 x 10⁶ sperm per ml, then rediluting it in a modified Caprogen diluent. This allowed inseminate dose rates of between 6 and 8 x 10⁶ SPD while maintaining fertility. RDF was stored cryogenically in 0.5ml French straws each containing 25 doses. It was thawed and rediluted to 16 x 10⁶ sperm/ml or 8 x 10⁶ sperm/0.5ml inseminate. Thawing and redilution was performed in the laboratory prior to packaging and despatch [56].

Rediluted Deep Freeze enabled the Farm Production Division of the New Zealand Dairy Board (FPD which was LIC's predecessor) to collect and store semen from the highest ranked bulls during times of insufficient demand for LLL semen. The rediluted product was used to supplement LLL in times of highest demand. As it was at a lower sperm dose rate than the standard frozen product there was a direct benefit to farmers by delivering a higher average BW than would have been the case had the semen been frozen in single doses.

In the early 1980's researchers at FPD developed 'Sempak' which was a further modification of the RDF system. The inseminating technician performed the redilution in the field using a tube of diluent stored in a domestic freezer.

The advantages of the system were:

- ◆ Inexpensive storage. Ten doses of semen were held in one 0.25ml straw. The cost per dose for the diluent packaging and storage were negligible.
- ◆ Servicing of remote markets. Sempak was largely used on the West Coast of the South Island of New Zealand at a time when transport services were unreliable and daily demands from a burgeoning dairy industry were unpredictable.
- ◆ Economy. Fewer sperm were used in comparison to servicing the market with single dose frozen semen. Also, technician's supplies were replenished every fourteen days instead of every three days with LLL thus saving substantial transport and logistics costs.
- ◆ Reliability. The system guaranteed a semen supply in an area that had unreliable, weather-prone transport systems.

Vishwanath and Pitt conducted RDF field trials in the mid 1990's using cryogenic blood storage bags for frozen semen storage. Up to 1000 doses could be cryogenically stored in a single bag, saving straw costs and storage space. There was no loss of fertility at 6-8 million SPD when used on the day after thawing and redilution [57].

Based on Caprogen's success as a semen diluent it was also assessed as a medium for freezing semen. The enhanced lifespan of semen at room temperature in Caprogen did not translate into a cryoprotective medium for frozen semen. It was found that Caprogen had deleterious effects upon semen when the temperature fell below 5°C and almost fatal effects upon freezing. One component of Caprogen that is considered essential to its

full effectiveness on ambient temperature semen survival crystallizes out of solution at low temperatures. This causes high levels of membrane damage and commensurate loss of survival. Although Caprogen is not a good freezing medium, it was obvious that Caprogen did enhance survival and fertility once semen had been rediluted post-thaw at temperatures above 5°C.

2.8 Conclusion and Recommendations

Livestock Improvement Corporation's liquid semen technology permits wide usage of its best bulls as the benign nature of the process allows for very low sperm dose rates (in comparison to frozen semen) while maintaining fertility. However, LLL has a limited usage life of four days and cannot be specifically targeted to individual cows.

The advantages of frozen semen are in the ability to store semen harvested in times of low demand and to deliver it to specific animals when and where it is required. However, frozen semen requires twelve times the number of sperm per insemination to maintain fertility in comparison with liquid semen.

In dairy breeding schemes genetic progress is optimised when elite proven bulls provide the majority of fertile matings. The key driver in gaining more inseminations per ejaculate is lowering sperm dose rates per insemination. LIC's research and development thrust has been to lower dose rates and extend the life of its liquid semen systems and to lower dose rates in frozen semen.

Shannon's RDF technology proved that semen frozen at high concentrations and rediluted in to Caprogen could maintain fertility using sperm numbers that fell between the liquid and frozen dose rates. Further research was required to gather the advantages of the RDF system into a single frozen dose.

In the following chapter the industry needs and constraints are outlined and the context in which product development is set is detailed. In

particular, the need for high fertility and the widespread dissemination of superior genes is demonstrated. The constraints imposed by costs and the need to maintain the use of the IMV 0.25ml frozen semen straw are also elaborated.

3 Industry needs and constraints for frozen semen product

In order to understand the needs and constraints imposed upon artificial breeding systems using frozen semen it is necessary to review the current technology and to examine the contextual framework in which this technology operates.

This chapter deals with the unique set of needs and constraints imposed on commercial artificial breeding companies in New Zealand. Within the product development sequence it is imperative to have clear objectives to meet industry needs. In this particular product development all criteria had to be met as the failure of any one of the three major points made below would have rendered the product inoperable.

The dairy cattle artificial breeding industry in New Zealand has at its core the following prerequisites:

- ◆ **High fertility.** This is essential for farms to maintain synchronicity of the breeding cycle with the spring peak in grass growth for feedstock. As stated in previous chapters, New Zealand's pastoral farming systems depend for their efficiency on the supply of grass that reaches its peak growth in the spring period, September to December. The nutritional demands for lactation are most efficiently met by synchronising calving with the spring flush of grass growth. To ensure that a high proportion of the herd will calve during this time fertility must be optimised, as empty cows do not lactate in the following spring. Fertility equivalent to a normally fertile bull is considered acceptable. This normally falls in the range of 65 to 70% non-return rate (NRR).
- ◆ **Widespread usage** of sires proven to pass on superior genes for production, health, fertility and tractability traits. As semen is the most efficient means of distributing desired genetics, it follows that the greatest possible use of the superior sires will lead to the fastest possible genetic gains. LIC's liquid semen systems have made genetic gains of 1% per year possible over the entire dairy population using LIC bulls [10]. However, as outlined in previous chapters,

constraints of supply and demand also indicate the need for high efficiencies in frozen semen technologies.

- ◆ **Low costs.** The true cost of all the components in a breeding program are reflected in the cost to the farmer of breeding replacement milking stock. The progeny must be of higher genetic merit than the sires and dams for genetic progress to be made. The costs for breeding and proving bulls, straws and storage and distributing semen are fixed. Savings made in semen processing by technological development are in the components of the diluents and labour. The major saving to be made is in lowering the sperm dose rate as this garners more doses per ejaculate processed and makes more genetic progress within the national herd.

Points two and three are largely addressed by lowering sperm dose rate. However, maintaining animals with high fertility must be the primary consideration. Without fertility, none of the other benefits can be accrued.

3.1 Frozen semen composition and costs.

Livestock Improvement Corporation uses a system developed in the 1970's by Foote et al for its core frozen semen product [58]. The specifications in Table 3.1 consist of the following key functions:

Table 3.1: Tris based frozen semen diluent components and their function

Function	Constituent	Parts/litre	Molar concentration
Buffer	Trishydroxymethylamine	24.3	200.6 mmol
	Citric Acid	14.0	66.6 mmolar
Cryoprotective	Glycerol	62.8	681.9 mmolar
	Whole egg yolk	200	
Metabolic substrate	Fructose	10.0	55.5 mmolar
Antibiotic system	Penicillin	100 x10 ³ international units/ml	
	Streptomycin	100 x10 ³ international units/ml	
Antioxidant	Catalase.	0.3	

The diluent is cooled to 4° C before it is saturated with nitrogen gas to exclude oxygen.

Table 3.2 shows the complete componentry of the standard Tris-based system compared to the new single dose product described in this thesis, including the sperm, seminal plasma and water. The standard system in use through many first world countries is based on the Tris recipe.

Table 3.2 Comparison of inseminate components, Tris vs new process

Tris components		New process components	
	wt%		wt%
Sperm	1.0	Sperm	1.0
Seminal plasma	3.0	Seminal plasma	3.0
Water	68.5	Water	70.0
Tris buffer	2.2	Sodium Citrate	2.9
Fructose	0.9	Glucose	0.3
Citric acid	1.2	Citric acid	0.3
Glycerol	5.6	Glycerol	8.0
Antibiotics	0.2	Antibiotics	0.2
Egg yolk	17.8	Egg yolk	15.0

The critical specification is the sperm dose rate. Most producers throughout the world use between 15 and 25 x 10⁶ SPD. LIC is able to take advantage of the intrinsic ally high fertility of the New Zealand dairy herd and is able to use 12.5 x 10⁶ SPD to maintain similar fertility to worldwide standards.

The two technologies depicted above share many common, fixed costs of processing. The major differences are in the costs of the chemical components where the new system compares favourably largely due to the lowered cost of the buffering system, (Tris vs sodium citrate/citric acid).

3.2 The effect of sperm dose rate on fertility

There is a direct interaction between the number of sperm in an insemination and its fertility. Individual bulls also have their own intrinsic fertility, which manifests at different levels according to dose rate. This bull by dose rate interaction is far more pronounced in frozen semen than it is in LIC's LLL product [59]. Each bull has a level of fertility that is affected by the number of sperm available for conception. Some bulls'

semen withstands the rigours of freezing and thawing better than others [1], [60]. This is manifest in their NRR.

There is insufficient commercial pressure on semen producers to investigate each bull's optimal sperm dose rate to maximise fertility. The resources for this are not cost effective within the productive lifetime of most bulls. To grow a bull, then test his fertility over a range of dose rates to assess his optimal sperm per dose level takes almost as much time as he is likely to be in semen production. Therefore, product is processed at a generic sperm dose rate that will give maximum fertility for all the bulls meeting the producers' quality control criteria. That is, they use the lowest sperm dose rate that gives the highest fertility for all bulls, rather than customising dose rates by bull.

The graph (Figure 3.1) below is derived from work by Den Daas et al showing the relationship between sperm dose rate and fertility in frozen semen [1]. It shows a precipitous rise in fertility at dose rates up to a point between 1×10^6 and 5×10^6 SPD where it begins to level off. There is a less pronounced rise up to a maximum fertility level of 65 – 70% NRR at around 15×10^6 SPD. As stated previously, this point varies for each bull. Semen producers maximise fertility for all the bulls they process by using the dose rate at which they know all bulls will reach maximum fertility.

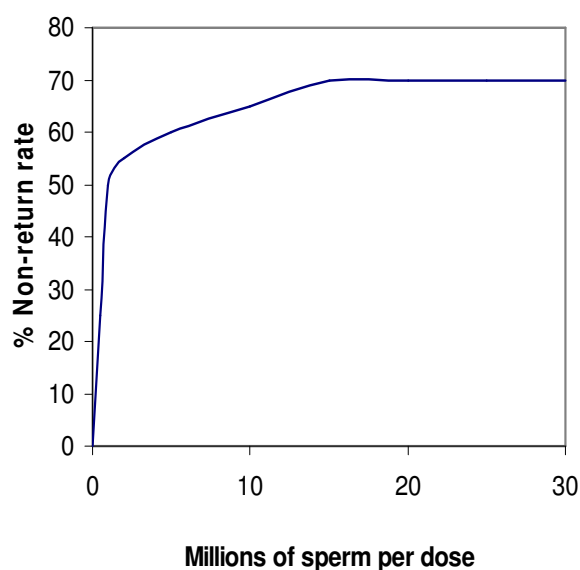


Figure 3.1: The relationship between sperm per frozen/thawed dose and fertility in bovine artificial breeding. [1]

For most semen producers this point is fixed between 15 and 25 x 10⁶ SPD. It can also be seen from this graph that in spite of rising sperm numbers fertility is not enhanced beyond the intrinsic maximum that is determined by the female population. Above that point there is no gain to be made from using more sperm in a dose.

3.3 LIC's standard (Tris-based) frozen semen processing procedure

LIC's standard frozen semen product is based on the above recipe and uses the following procedure:

- ◆ Semen is added directly to approximately three times its own volume of diluent at 31° C.
- ◆ The semen plus diluent admix is cooled to 4° C over 30 minutes.
- ◆ The product is extended to its final sperm concentration with further diluent.
- ◆ Product is equilibrated for 3.5 hours to allow from the time of primary dilution.
- ◆ It is then packaged into 0.25ml IMV type straws for freezing and storage.

This is a standard frozen semen diluent system, used worldwide by many semen producers. It is derived from work by Foote et al that sought to upgrade the buffering systems being used at that time to process semen for freezing [61], [58], [62].

Any attempt to lower the dose rate to gain more doses per ejaculate must address the question of fertility. It was against the above specification and the critical need to maintain fertility that this project would be judged.

3.4 Historical background to industry needs

Farmers have selectively bred cattle for thousands of years. The tractable, fertile, robust, healthy, efficient animals farmed in the first world today are the result of selection from the wild Auroch populations of Eurasia that has transformed the species to fit man's requirements.

Until recently farmers could only select animals to breed their next generation of stock from a limited supply of potential dams and sires resident in the close vicinity of the breeding unit. Selection criteria were largely unmeasured and relied on the farmer's eye and his particular needs.

The recognition of the sperm and egg as the carriers of the next generation's genes was a breakthrough that allowed the concept of artificial breeding to follow. Once it was established that a single sperm and not the entire ejaculate was responsible for a fertilization event, the possibility of splitting an ejaculate to gain more pregnancies from a bull with superior breeding traits was explored.

3.5 Early attempts at artificial breeding

Early experiments in artificial breeding consisted of allowing a bull to serve a cow naturally, manually retrieving some of the ejaculate and transferring it to the anterior vagina of another recipient cow. A refinement of this technique used a sponge inserted into the vagina to trap sperm. This was later retrieved and the sperm recovered for further use. While not spectacularly successful, some pregnancies resulted and the possibility of improving the technique was explored. The development of the artificial vagina allowed the entire ejaculate to be harvested and extended.

Advances in the knowledge of the physiology and anatomy of the cow saw experiments in trans-cervical insemination using longer devices to transit the cervix to the posterior uterine cavity. A further major advance was made by the realisation that the manipulation of the inseminating

device through the cervix could be facilitated by palpation through the rectal wall.

Observation and improved detection of the external signs of oestrus lead to optimisation of insemination times. It became clear that the best method of raising genetic progress was to select for traits passed on by the bull as only the bulls' gametes could be subdivided and used over multiple matings. Research into semen preservation and dilution has been going on since the 1930's.

3.6 The economic need for genetic progress

The need for more efficient utilization of the genetics from dairy bulls is well established. Early cattle breeders recognized that superior progeny could be produced, and herds improved, by restricting matings to those bulls passing on genes best suited to the demands of their particular farming practices. That is, breeding selectively for traits that made more efficient use of land, feedstock, available farming technology and cultural purposes.

Cattle have been bred for multi-purpose roles: for meat and milk, for draught duties and for production of specific body components such as hide or horns. The tractable, docile dairy cattle present in modern herds are also the result of selection for biddable behavioural traits. Conversely, familial propensities for aggression are bred into animals used for bullfighting and rodeo.

In modern dairy practice, the efficient conversion of food into milk components is the paramount selection criterion [63]. Dairy animals have been bred to utilise different feedstock available in various parts of the world. These breeding practices usually also take into account the farming practices used to provide the feed and to accommodate the herd. For instance, the feedlot farming practices utilized in North America require large-bodied animals with wide hooves and tractable behaviour. These animals are housed indoors on concrete floors for much of the year and do

not have to forage for food. Their wide hooves allow the animals' weight to be better distributed on the hard floor where the cow is required to stand for most of her working life. North American cows are bred to efficiently convert the total mixed ration (TMR) type diet into milk products. This feedstock is carried to the cow and as there is no need to walk, forage or compete for food more energy can be directed into lactation. As there is a direct correlation with animal size and lactational capacity, feedlot-managed cows have been bred for size as well as the ability to survive in a closed-barn system of farming. These cows tend to be larger than pasture fed cows [64].

Grasslands farming, practiced in many southern hemisphere countries like New Zealand, Australia and Argentina requires an animal that can forage aggressively for pasture and keep good health outdoors. These animals tend to be lighter than the North American strain to conserve pasture and soil condition in wet weather. Their smaller size also ensures that a greater proportion of their energy intake is directed to lactation rather than body maintenance needs. Pasture farmed cows also need good hooves and legs, as they must be mobile to forage and to assemble for milking [65].

3.7 Fertility of cows under lactational stress.

Some mammals suppress ovulation while lactating. This survival mechanism ensures that the energy demands on the female do not overwhelm her by having to maintain a pregnancy while lactating. Modern cattle are able to ovulate and conceive while lactating heavily. This ability may have been the result of selective breeding for females showing this trait.

In seasonal, pasture based farming systems such as practiced in New Zealand this ability to ovulate while lactating is essential to the survival of the animal and to the economic viability of the farming unit. Any animal that does not conceive during the spring will not lactate the following year and represents a non-productive cost to the farmer. Non-pregnant or 'empty' cows are invariably culled at the end of their lactation. Farmers

cannot afford to carry the cost of feeding and maintaining an animal that will not produce. This has resulted in an extreme selection pressure for fertility in dairy animals in New Zealand. Only the animals able to conceive are allowed to survive. The non-pregnant animals are eliminated from the breeding stock. Over past 150 years since dairying became established in New Zealand this rigorous selection pressure has resulted in a highly fertile strain of dairy animals that is able to conceive, grow the calf and lactate efficiently and contemporaneously over several pregnancy and lactational cycles [66].

This contrasts with the feedlot farming practices in the northern hemisphere where there is no pressure to synchronise with seasonal feed growth cycles. Animals not conceiving immediately after parturition are commonly allowed repeat inseminations or matings to try to establish a pregnancy. As the food supply is constant and non-cyclical, farmers have been able to concentrate their selective pressure on milk production with a tolerance of lowered fertility [67]. It is common for feedlot cows to lactate for over four hundred days and some modelling has used five hundred days in anticipation of the combined effects of feedlot practices and the application of Bovine Somatotrophin (BST) hormone to extend lactation [68].

Within feedlot farming practices it is known that the stresses placed on the energy demands of lactating animals negatively affect reproductive performance. The higher yielding cows are more likely to have lowered fertility [69]. In the New Zealand pastoral farming context, the selection pressure from the culling of low-fertility animals has produced highly fertile animals. New Zealand farmers place high demands on their animals for both milk yield and reproductive performance.

Farmers expect that all the other links in the reproductive chain operate optimally to maximise pregnancy rates, calving rates and genetic gain. The most important part of this chain is the supply and correct placement of fertile semen from bulls proven to be genetically superior for milk production and reproductive traits in their daughters.

3.8 Cattle Breeding requires long-term, strategic predictions

Modern dairy selection practices strive for efficient conversion of feedstock to milk products [70]. To achieve this requires foresight in several key areas:

- ◆ Trends in the milk components market; for example, the fat/protein ratio.
- ◆ Trends in the total animal product markets; for example, dual purpose (meat and milk) versus single purpose (meat or milk).
- ◆ Land usage trends; for example, change of feedstock brought about by social or environmental pressure on land management.
- ◆ Changes in product transport costs; for example, penalties for high water content in milk.

The breeding of elite sires is a long-term strategy. The time from birth to proof of a potential sire is four years, made up of:

Year 1: Birth, maturation to puberty, sire-proving scheme (SPS) matings.

Year 2: Lay-off during which SPS heifer calves are born and reared

Year 3: Lay-off during which SPS heifers are first mated, gestate and calve.

Year 4: Lay-off during which SPS cows produce their first lactation.

At the end of this time a comparison of the bull's daughters' production efficiency is made with her herd-mates. The bull's genetic contribution to his daughter's efficiency can then be assessed. He will then be either used extensively for artificial breeding or culled as genetically inferior.

This selection process is so rigorous that companies like LIC are able to discard 95% of bulls proven as unsuitable for breeding. The selection criteria also take into account traits such as health, fertility, manageability

and body conformation. The result is an animal suited to farming practices and market requirements predicted five years earlier.

3.9 Overall genetic gain in the New Zealand Dairy population

LIC's selection practices have resulted in a genetic gain (in terms of production) of one percent per year for the last forty years in the NZ dairy cattle population [71]. While selection criteria have changed in some respects, (protein production is now favoured over fat), the over-riding consideration is the breeding of a tractable, healthy, fertile animal that converts grass to milk solids more efficiently than the previous generation.

Without the statistical comparison with herd-mates, (that is, the bull proof), farmers could be using sires that actually lower production and profit, by passing on inferior genes to their daughters.

3.10 Breeding objectives for efficiency in farming business practices

Grasslands farming has become a commodity driven business that supplies milk to large scale processing facilities for conversion into bulk quantities of higher value product. The world market for these products (butter, cheeses, milk powders, casein) is intensely competitive, relying on the economies of scale and cheap supplies of milk. Milk is the feedstock for the processing industries but it is the price of the feedstock for the cows that drives the ultimate cost of milk. In New Zealand there is no cheaper system of producing feed for conversion to milk than growing grass, which is harvested by the cows themselves. This contrast markedly with most First World dairying practices where heavily price subsidised rations are carried to the milking herd which is housed throughout much of the year.

Genetic gain is only one of the tools used by modern farmers to maintain efficiencies. Better pasture management, automation and advanced

veterinary care all contribute to more efficient farming practices and increased profitability. However, without directed and selective breeding, farmers risk actually losing production due to lowered conversion efficiencies passed on by inferior sires.

Dairy product commodity pricing is intensely competitive. New Zealand farmers are in competition with northern hemisphere dairy industries, which are only profitable due to subsidies. In the face of this competition they have striven for efficiencies to keep costs low. Selection for genetic gains for production is one of their most important tools for garnering these efficiencies. Artificial breeding is the means by which they incorporate these gains into their herds.

3.11 The need for frozen semen

Although LIC's flagship product is liquid semen, the limitations of its shelf life necessitate the use of frozen semen to back up the main product.

A variety of needs indicate the use of frozen semen:

- ◆ The need to match a specific bull with a cow. For a variety of reasons the farmer may not wish to mate a specific a cow with the bull/s supplied in liquid semen on the day she is in oestrus. Frozen semen allows the choice of a specific sire for a mating.
- ◆ Shortfall in liquid semen supply. Inaccuracies in the liquid semen demand predictions lead to shortfall in supply. It is impossible to predict exact daily demand for each farm as the ratio of cows presenting in oestrus can vary from nil to over 10% of the herd on any day during the spring. On rare occasions it is inevitable that there will be a shortage of liquid semen; frozen semen is the back up in this situation.
- ◆ Economies of servicing remote areas. Isolated farms in areas without dedicated public transport links are more economical to service with frozen semen as the need to refresh the semen

supply every three days drops to once in fourteen days, the storage life of a liquid nitrogen bank.

- ◆ Avoidance of inbreeding if the sire supplied in liquid semen form is too closely related to the recipient cow. LIC inseminating technicians are supplied with a hand-held data logger (DataMate) that is programmed with individual cow ancestry information. The device flags a warning when a proposed mating exceeds a “relatedness” limit set to avoid inbreeding. Technicians are supplied with frozen semen from alternative sires to use in this situation.
- ◆ ‘Genetic Insurance’ – the maintenance of a supply of elite genes held in case of a disaster such as a major disease outbreak. A disease outbreak or any other disaster, which disables the bull or the breeding facility, or restricts product movement, would prevent use of the semen for future breeding of sires. Breeding companies take advantage of the unlimited storage life of frozen semen to keep a supply from their top sires isolated from the production facility in case of the loss of either the sire or the facility.
- ◆ Matings outside the main seasonal demand when liquid semen production is uneconomic. LIC operates a ‘break-point’ where the cost of transport and servicing out-weighs the cost benefits accrued from liquid semen production. It is more efficient to supply low numbers of matings with frozen semen from a regional supply base than to process and transport small quantities of liquid semen from the central production facility.
- ◆ The ability to sell semen internationally. Frozen semen is the mainstay of AB throughout the rest of the developed world. LIC has an active international marketing division to take advantage of the unlimited storage life of frozen semen.
- ◆ The ability to import fresh genetic material. Breeding companies try to avoid inbreeding by importing sires representing different strains within a breed to add genetic diversity. When breeding

objectives change it may also be necessary to import new genes to cater to the new target.

- ◆ Quarantine. The short shelf life of liquid semen precludes post-collection quarantine to incubate semen-borne disease. Many First World countries operate a system where semen is held in quarantine after freezing for a prescribed period for any disease to manifest in the sire or his contemporaries. New Zealand is able to operate a fresh semen system because of its isolation and strict border controls on exotic cattle diseases.

3.11.1 Infrastructural investment in frozen semen

Livestock Improvement has a large and comprehensive infrastructure developed around the supply of frozen semen to its clients. Annual use of liquid nitrogen is greater than 500,000 litres and LIC owns 1500 portable liquid nitrogen banks, each capable of holding 800 – 2000 straws.

LIC also maintains frozen semen stocks at two sites, geographically well separated in case of a disaster in one or the other region. The stocks are held in more than fifty large vacuum vessels each capable of holding 200,000 straws. Stock control over inventory, ordering and despatch are all computerised. However, due to the harshness of the environment under liquid nitrogen, machinery cannot be used for transferring and handling semen stocks. It is a manual process, performed by teams of handlers in the months prior to the spring.

3.11.2 Artificial breeding systems differ according to context

First World dairy farming practices strive for efficiencies in feed production and conversion. The economies of modern large-scale farming necessitate that animals are bred to convert the cheapest available feedstock to dairy product in the most efficient manner available. Animals are tested for output, graded and judged, then

culled as more efficient animals are bred. Coupled with this is the need to start and maintain pregnancy to induce the next lactation and to breed replacement stock.

To meet the need for the most widespread dissemination of genes from elite sires systems of artificial breeding have evolved within the economic constraints of specific local dairy farming systems.

In farming systems where matings are spread through the year the method of choice for AB is by frozen semen. This allows the farmer to choose the specific bull required and to time the mating to match the cow's fertile period. Many farms exclude live bulls altogether from their systems. Semen is thawed and used when necessary, allowing for exact breeding management without the necessity to feed, house and manage bulls.

Bulls bring a variety of problems to farmers:

- ◆ Feeding requirements
- ◆ Behavioural problems.
- ◆ Veterinary requirements.
- ◆ Housing requirements.
- ◆ Cow health (injury, disease).
- ◆ OSH considerations.

Indeed, LIC has calculated that it is economically advantageous to NZ dairy farmers to use AB in the place of bulls *to circumvent the previously stated list of problems alone*. Coupled with the genetic gains made from sire proving and selection AB becomes even more attractive to dairy farmers.

As an example of the contextual usage of artificial breeding systems, most first world AB uses the 'French' straw developed by Cassou et al at IMV in l'Aigle, France. Poorer nations have developed systems where semen is frozen in unpackaged pellets on

metal trays over liquid nitrogen. This system invariably leads to lowered efficiencies due to loss of semen quality, potential bacterial and viral contamination and the lack of individual dose identification. However, costs are lower and such systems serve the economic needs of those nations well.

Glass ampoules containing frozen semen were used before the development of the straw based systems. The low surface area to volume ratio of the ampoule and consequent low heat transfer rate lead to high sperm losses and the necessity for high sperm numbers per inseminate dose to compensate.

Minitub of Germany also developed a straw based system for frozen semen using a similar straw to the present liquid semen straw used by LIC. Initially this was sealed by forcing steel beads into the ends of the straw. This system has not seen large uptake worldwide.

The world picture for artificial breeding is of a market dominated by frozen semen using the 'French' style of straw pioneered by IMV and copied in later years by various imitators. Most first world cattle breeders have large infrastructural investments in place to accommodate this system.

3.12 The need for liquid semen in seasonal pastoral farming systems

Within the farming practices in widespread use in New Zealand, the dominant economic driver is the production of cheap feed from pasture. No other system of feeding grazing animals has been found that can compete economically with the cattle-harvested, pasture-grown grasses that thrive in New Zealand's temperate, oceanic climate. Much study in New Zealand has been undertaken to compare farming systems such as 'total mixed rations' (TMR), 'cut-and-carry' and alternative feed plants (for example, maize, millet) with grass based farming. The results have invariably favoured grass as the major feed supply. Winter feed to

supplement pasture is harvested from excess spring and summer grass growth and stored as hay or silage.

This farming system is driven by the seasonality of the grass growth cycle. Grass growth peaks during the spring when soil moisture is high, rainfall is plentiful and soil temperatures rise above species-specific thresholds for optimum growth. To take advantage of the grass growth the farmer coincides his herd's calving and therefore, lactation with the peak. Just as the herd enters its most intensive period of lactation there is plenty of food to fuel the metabolic demands of the animals.

All other farming activities are driven by this one economic constraint. As the timing of calving and lactation is critical, so too is the timing of matings. Whether this is to be achieved naturally or artificially, there is a peak in the demand for this essential farm service.

Pat Shannon and his co-workers at the New Zealand Dairy Board recognized that the demand for semen supplies during the spring in New Zealand could be more efficiently met by lowering the sperm per dose rate per insemination. Frozen semen technology had reached a limit of fertility when sperm dose rates were used below 25×10^6 SPD with the technology available in the early days of artificial breeding. Shannon saw that New Zealand would need huge reserves of bull power to meet the peak spring demand at that dose rate. The answer was to extend the best bulls' ejaculates further by lowering the sperm dose rate per insemination. This was achieved by avoiding the stresses on the sperm of freezing and thawing and maintaining the sperm in an unfrozen state for as long as possible.

Major advancements were made in the first ten years of the development of Shannon's ambient temperature diluents. Dose rates were progressively lowered to 2×10^6 SPD without affecting fertility. The useful life of the diluted semen product was extended to four days. The establishment of a transport infrastructure that delivered the product within twenty-four hours of collection and dilution allowed a useful life of three days after

which the product was replaced with a fresh batch. Widespread usage of only the very best sires was made possible using 2×10^6 SPD.

Further development of the diluents has lowered the dose rate to 1×10^6 SPD [59]. Under this semen dilution regime some sires are capable of more than 250,000 inseminations per year, the majority of which are made over an eight-week period during the spring.

Without the overheads incurred in a large-scale liquid nitrogen-based distribution system (for frozen semen) the liquid semen based system has produced genetic gains for New Zealand farmers on a par with the major dairying nations of the world at approximately one tenth the cost of a comparable frozen service. This is reflected in the cost of breeding replacement dairy stock in New Zealand, which varies around ten percent of the cost in North America or Europe.

Without the ability to achieve widespread usage of only the elite sires genetic progress in the New Zealand dairy population would not have been as rapid. The capability to use a limited number of sires is so highly developed that LIC has to limit usage in some cases to prevent over-representation of some genetic lines in the population. That is, some bulls are not used to their full capacity, as the system could produce so many doses the genetic base of the population would be adversely narrowed.

3.13 Economic benefits of lowered dose rates in A.B.

3.13.1 Liquid (ambient temperature) semen

The widespread dissemination of superior genes has raised the productive capability of the national herd. The extent of this improvement has only been possible through artificial breeding; by using the semen from superior bulls at ambient temperature it is possible to use sperm dose rates unequalled in the world, while retaining fertility. It was obvious that lowering the sperm dose rate was the key to enhancing the spread of superior genetics in New Zealand's seasonal, grasslands based dairy industry. The use of

liquid semen gave New Zealand farmers large numbers of inseminations from the very best bulls at the lowest cost.

3.13.2 Frozen Semen

The same need arises in frozen semen technology. While it is not the mainstay of AB in NZ it is the only alternative in the situations outlined previously, precluding the use of LLL semen. By lowering the dose rate in frozen semen, every ejaculate collected in the 'off-season', (that is, the non-liquid semen portion of the year) has more widespread usage.

Farmers benefit from lowered dose rates in frozen semen in the following ways:

- ◆ Increased availability of the best bulls gives better choices when there is a need to match a specific bull with a cow.
- ◆ When the liquid semen supply runs short there is increased availability of the best bulls' semen in frozen form.
- ◆ When remote areas are more economically serviced with frozen semen there is increased availability of the best bulls.
- ◆ 'Genetic Insurance' – the processing of a supply of elite genes held in case of a disaster is more economic as fewer ejaculates are needed to meet requirements.
- ◆ When liquid semen production is uneconomic outside the main seasonal demand, more frozen semen from elite sires is available.
- ◆ More semen is available for international sales that eventually subsidise the local product price.
- ◆ Opportunities exist to sell or license the technology for lowering dose rates to overseas semen producers. This

benefits farmer shareholders by garnering a return on investment and may also subsidise local product prices.

Since the 1950's when AB began in NZ LIC and its predecessors have been able to decrease the average number of daily semen collections from more than 100 in the spring to a maximum of 40. In addition, the off-season daily collection rate has fallen from over 30 to an average of 10. This is due to the lowering of dose rates per insemination using both liquid and frozen technologies. The overall effect has been to create more doses per ejaculate. This has led to a gain in the average breeding worth of the semen supplied and a commensurate lowering of overheads.

Semen frozen under liquid nitrogen appears to have an almost unlimited storage life. At -196°C molecular activity is so low that chemical changes are almost non-existent. There may be some effects from background ionising radiation but these have not been studied in depth or quantified. Semen frozen for forty years is still viable and fertile [72].

3.13.3 The IMV straw and the industry infrastructure supporting it

Almost all artificial breeding in cattle throughout the world uses the IMV frozen semen straw. Developed for commercial use by Cassou, Walbaum et al in the late 1960's, it has become the industry standard for holding semen while frozen and as the delivery mechanism for deposition into the cow.

The 135mm x 2.0mm IMV straw has been developed to maintain high sperm recoveries after thawing while presenting in a commercially viable package. This has led to widespread uptake and success in cattle AB. The surface area to volume ratio of the straw gives sufficiently high heat exchange rates to allow sufficient sperm to recover after thawing to make AB with frozen semen cost effective [41].

An industry infrastructure has been created to handle the IMV type of straw configuration. Storage and handling in liquid nitrogen requires specialized inventory systems, as the environment is very harsh to most materials. Lubricants fail, metals and plastics become brittle, machinery seizes. The only way to manipulate frozen stocks is by hand using the simplest tools such as forceps, tweezers and scoops.

Liquid nitrogen storage vessels are compartmentalised to accommodate multiple layers of straws. Further breakdown of batches of straws is into specialist 'goblets' made from Ethyl Vinyl Acetate, one of the few plastics that retains some flexibility at cryogenic temperatures.

The inseminating technician's equipment is also made to accommodate this type of straw. The dimensions of all the surrounding equipment are based on handling and using the straw. Indeed, the size of the technician's portable LN₂ banks is also determined by the required straw capacity.

This infrastructure represents a large capital investment to an AB company. The industry has matured into using this technology and would be loathe to change without a major economic benefit.

Hence the need to develop improvements that already fit into the existing systems. Any brief to improve semen preservation technology comes with the proviso that the existing packaging, handling and usage criteria are retained. Any change would be expensive and disruptive.

3.13.4 Freeze damage to sperm

At least twelve times more sperm have to be placed in a frozen straw than a liquid semen dose. The stresses evoked during freezing and thawing result in the loss of motility in half of the sperm in the straw. Semen from different bulls and indeed from

different ejaculates reacts variably to freezing. The progressive loss of heat from the straw and the glass transition point all impact on sperm survival [73].

Freeze damage to sperm is not simply confined to the motility mechanisms. Various other physiological processes and features are affected:

- ◆ Acrosome reaction. The acrosomal region of the sperm has to undergo a maturation process post-ejaculation to ready the sperm for binding to the zona pellucida surrounding the egg. This process is termed the 'acrosome reaction'. After freezing and thawing nearly all sperm in a batch are acrosome reacted [74].
- ◆ Acrosome damage. The acrosome is damaged in many frozen thawed sperm, which renders them inviable. These sperm may retain motility and the integrity of their somatic membranes but they are not capable of fertilization [50].
- ◆ Capacitation. Freezing and thawing can also damage the processes of post-ejaculatory maturation collectively called capacitation.
- ◆ Chromatin damage. The DNA content of the nucleus can be damaged by freezing and thawing. Sperm with undamaged motility and egg binding mechanisms may still be inviable as their chromatin structure is damaged [75].
- ◆ General membrane damage. The sperm membrane can lose its integrity after freeze/thaw. This leads to loss of fertilizing ability as a variety of processes are compromised [45]. The eversion of membranes exposes binding sites for leucocytes present in the reproductive tract. These actively phagocytize sperm, which are quickly removed from the tract [76].

- ◆ Mitochondrial sheath/tail sheath damage. Damage to the sheath surrounding the mitochondria and the tail structures leads to loss of motility or defective swimming. Tail damage is often manifested in morphological changes or in unusual swimming patterns.
- ◆ Actin/myosin damage. Damage to the muscle-like swimming mechanisms in the tail can also lead to loss of motility or defective swimming.
- ◆ Mitochondrial membrane/mechanism damage [77]. Changes in the mitochondria membrane disrupt energy-generating mechanisms and lead to loss of motility. It has also been postulated that the mitochondria act as a sink for radical oxygen species (ROS) generated in the dead and dying sperm in an ejaculate.

Cumulatively, the points above add up to the need for greater numbers in a frozen inseminate in comparison to unfrozen to achieve comparable fertility. Despite this, the benefits of frozen semen as outlined above, still present it as an economically sound system in the dairying nations where it is applicable.

3.13.5 Quality control parameters for frozen semen

Natural variability in the quality of semen and its reaction to freezing and thawing indicates that there will be variation in the numbers and viability of thawed sperm. A set of laboratory test parameters has evolved to guide the semen producer in deciding on the viability of a batch of frozen semen. However, it is accepted that no strong correlation exists with any one or combination of parameters that can be measured in relation to post-thaw semen quality [78]. Rather, the producer relies on a bull's past performance in the field and any deviation downward from his normal quality is taken to indicate possible fertility problems.

It is accepted that there is variation within a bull's fertility and that there is also variation between different bulls' semen in its reaction to freezing. Different bulls' semen also reacts variably to alternative extender systems; optimal extender performance for one bull's semen may not suit another.

Livestock Improvement Corporation has relied on the incubation or heat stress test as its main indicator of the viability of a freeze thawed sample. A pooled sample of the contents of four straws is incubated in a 3ml glass agglutination tube at 37°C for a set time. Semen quality is assessed immediately post-thaw and again at a time that is determined by the extender system being used.

The expectation is that the semen will deliver fertility comparable to natural mating. That is, a normally fertile bull will achieve a pregnancy in between 65 – 70% of matings. Diluted, frozen/thawed semen is adjusted for sufficient sperm numbers to also achieve this fertility level. Fewer sperm will lower the fertility of the insemination. However, the effect is asymptotic; more sperm will not achieve a gain in fertility as the upper limit is determined by factors in the female.

3.14 The benefits of dialysis and post-thaw redilution on Semen

The major portion of an unprocessed ejaculate is seminal plasma, which has several functions:

- ◆ As a carrier medium for sperm to transport them from the male and through the female reproductive tract.
- ◆ Substrate supply. Sperm do not carry sugar for energy requirements and must extract it from the surrounding medium.
- ◆ Antibiotic effects. Low molecular weight peptides in seminal plasma act as detergents on bacterial membranes. They also have a negative effect on sperm membranes and are partially responsible for the high attrition rate of sperm post-ejaculation.

- ◆ Buffering against pH change. Sperm operate optimally in a narrow pH range and seminal plasma is buffered to maintain this.

The above points show seminal plasma to be both beneficial and detrimental. For the commercial semen producer the negative effects of seminal plasma on longevity and post-thaw recovery are the most profound. It is known that seminal plasma binds to the outer sperm membrane and acts against sperm, shortening their lives and making them more prone to phagocytosis in the female tract.

Egg yolk competes for binding sites on the sperm membrane and offers some protection from the effects of seminal plasma. Sodium citrate based diluents disaggregate seminal plasma protein moieties to render them dialyzable.

It is impossible with the present technology to mitigate all the effects of seminal plasma on semen preservation. However, some modification to seminal plasma's effects is possible by disaggregation of peptides and dilution and/or dialysis of the products away from the sperm [79], [47], [48].

Shannon et al developed a dialysis system for semen to minimise the effects of seminal plasma. Dialysis membranes of 12kda mwco were used in conjunction with sodium citrate based diluents to effectively separate sperm from unbound seminal plasma proteins. When this system was coupled with egg yolk based diluents as competition for seminal plasma binding sites on the sperm membrane it was possible to produce a commercially viable frozen semen product at dose rates well below what was the industry standard of the day. This product was named 'Rediluted Deep Frozen' semen (RDF) [56].

RDF was used extensively in the seventies as a laboratory rediluted product to supplement the liquid semen supply in periods of high demand. Product was stored in 0.5ml French straws, thawed in the lab and rediluted into a modified Caprogen. This was issued in sealed glass test tubes. Inseminating technicians used a disposable polystyrene pipette to

withdraw a 0.5ml dose from the tube and to perform the insemination. The semen was used over the subsequent three days at 5×10^6 SPD. This compared favourably with frozen semen systems in use at the time, which were using 25×10^6 SPD with only a 10-minute window available post-thawing before insemination.

3.14.1 Glycerol Infusion by dialysis

A further advantage gained by the dialysis system developed for semen by Shannon was that potentially toxic cryoprotectants could be gradually infused. Osmotic shock is a well-known deleterious effect that occurs when cells undergo a rapid change in the osmolarity of the medium. Glycerol based diluents were found to be more effective when the level of cryoprotectant was raised gradually. For this reason, semen is commonly diluted initially into a non-glycerol diluent for cooling to processing temperature. Standard production methods include a progressive addition of the glycerol fraction, after cooling, over a time span determined to minimise the effects of the change in osmolarity. Generally this addition has been over at least one hour with a further equilibration period to ensure equilibrium within the cells.

The dialysis technique further slowed the infusion process. The glycerol addition occurred progressively over the concentration gradient and smoothed the transition to the optimal cryoprotectant level. It eliminated the step-wise changes in cryoprotectant concentration wrought by the addition of discrete aliquots of glycerol containing diluent. This was found to enhance post-thaw sperm survival and was one of the contributing factors permitting the lowered dose rates successfully used for the RDF technique.

3.14.2 The benefits of post thaw redilution

The RDF system also encompassed all the benefits derived from the liquid semen diluent developed for LIC's ambient temperature semen products. The redilution into Caprogen enhanced the post-

thaw life span of the semen to the point where it could be used over the 72 hours subsequent to thawing and redilution. The membrane stabilisers and antioxidants in Caprogen all enhance the longevity of the semen in comparison to simpler diluents.

3.14.3 Summary of the Rediluted Deep Frozen semen technology

The benefits of the RDF system can be summarised thus:

- ◆ Dilution into a sodium citrate based medium that disassociated spermicidal seminal plasma proteins to dialyzable proportions.
- ◆ Dialysis removal of spermicidal peptide moieties.
- ◆ Dialysis infusion of cryoprotectants
- ◆ Freezing into straws or bags. Each package had high surface area to volume ratios to enhance heat transfer.
- ◆ Redilution into Caprogen, which maximised post-thaw survival.
- ◆ Fertility comparable to fresh product, albeit at 2-3 times the sperm dose rate per inseminate.
- ◆ Sperm dose rates one-third to one-half the single dose frozen regime at the time.

3.15 Post-thaw semen survival

Sperm have to retain their ability to fertilize post-thaw to be of use to the producer and the farmer. This implies the retention of several factors, each of which is essential to maintain fertility:

- ◆ Motility. The sperm have to make their way from the inter-uterine deposition point to the egg. Loss of motility implies loss of fertility.
- ◆ Membrane integrity. Any damage to the cytoplasmic or mitochondrial membranes leads to rapid loss of viability.

- ◆ Ability to undergo capacitation and acrosome reaction. Both processes are necessary to prepare the sperm membrane for fusion with the egg. Loss of these abilities also implies loss of fertility.
- ◆ Cytoskeletal integrity. Freeze damage is sometimes manifested as a change in the gross morphological aspects of the sperm. Changes can be seen as distortions of various features of the sperm: tail recurving, loss of acrosomal membranes, membrane cratering, mitochondrial sheath damage can all be detected microscopically. Tail recurving in particular is indicative of damage to the sperm cytoskeleton and is often an external sign that there has been a raft of changes within the sperm, all of which lead to a loss of fertility.

Sperm that survive freezing and meet all the above criteria have the greatest chance of remaining viable and performing a fertilisation. However, the necessity to use twelve times as many frozen-thawed sperm than are required in an ambient stored inseminate implies that the majority of the motile, seemingly viable sperm are also damaged in some way that is not yet detectable.

3.16 Antibiotics

The action of sodium citrate and the simple dilution effects of semen extension dilute any antibacterial factors in the ejaculate to the point that it is necessary to add antibiotics to extenders [80]. By its nature, semen extender is a benign medium and will support bacterial growth. It is not possible to maintain sterility in diluted semen, as the raw product itself will always carry a bacterial load. Antibiotics have varying levels of toxicity to sperm. The problems of toxicity to sperm are compounded in non-frozen semen products as the sperm are in contact with the antibiotic at ambient temperatures for extended periods of time. Although the contact time is lessened in frozen products the added stresses of freezing and thawing can contribute to the deleterious effects of antibiotics.

The potential spermicidal effects of antibiotics indicate the necessity for toxicity testing of all candidate antibiotics. Many are indeed too toxic for consideration. AB companies balance the levels of antibiotics to maintain bacteriostatic conditions rather than trying to actually kill all the bacteria in an ejaculate. The levels required to sterilise an ejaculate would also kill most of the sperm.

The serious effects on sperm survival and fertility of a bacterial bloom within the extender necessitate that the manufacture of extenders is as clean as possible. This is to ensure any starting bacteria numbers are as low as possible. Companies like LIC prepare diluents under stringent conditions and test for contaminant loads. Despite this it is possible for resistant bacteria to gain a foothold in an extended batch of semen. For this reason LIC holds a stock of alternative antibiotics in reserve for treatment of both extenders and bulls. The use of liquid semen always carries with it the risk of resistant bacterial bloom.

3.17 Semen Assessment

Semen researchers and producers have developed tests to elucidate evidence of damage to the remaining motile, but not necessarily viable sperm.

- ◆ The simplest and most widespread test is the body temperature incubation test. A pool of the contents of thawed straws is incubated at 37°C and the survival time to a nominated end point of numbers left alive is assessed. LIC commonly uses survival time until 10% of sperm remain motile.
- ◆ “Live/dead” stains for microscopic studies. Sperm are stained with a combination of dyes that can transit damaged membranes and bind to DNA thereby indicating membrane damage. Counter stain combinations can be used to show intact membranes in a contrasting colour. Most commonly used is a combination Eosin/Nigrosin stain that shows up as either blue or pink for live and dead (membrane damaged).

- ◆ Flow cytometry. Combinations of fluorescent membrane transient dyes can be used in a flow cytometer to identify membrane damage sperm. It is also possible to identify DNA damaged sperm, mitochondria compromised sperm and to quantify stages along the capacitation-acrosome reaction pathways.
- ◆ Time exposure photographic quantification. A ten second time exposure is taken under a microscope using bright field illumination. The motile cells are counted as streaks while the non-motile remain as discrete points.
- ◆ Metabolic indicators. Various metabolic markers such as lactic acid production, oxygen consumption, sugar consumption have all been studied as indicators of metabolic function.
- ◆ Computer aided semen assessment (CASA). Software developed from movement tracking systems such as traffic control has been developed by various companies in an attempt to quantify several characteristics of semen motility. A microscopic field is recorded digitally and the software is able to distinguish between motile and non-motile sperm. Of the motile portion, analysis is possible on several characteristics:
 - ◆ Straight-line velocity
 - ◆ Curvilinear velocity
 - ◆ Head rotation frequency
 - ◆ Head displacement amplitude
 - ◆ Tail beat frequency
- ◆ Laser based semen analysis. Using techniques derived from laser guidance and direction finding systems, methods have been developed to assess most of the characteristics described above for CASA.

No significant correlations between any of the above listed tests have been made with fertility. Any one of the many parameters listed previously could have an effect on fertility. Taken alone, each attribute may

contribute to the overall fertilizing capacity of an ejaculate. However, there may be attributes not yet discovered, or not yet tested that contribute, and it would be erroneous to try to predict the fertility of a batch by any individually tested parameter. Similarly, no combination of the above tests has a significant correlation with fertility.

3.17.1 Visual semen assessment

The test most relied upon in commercial operations is the simple microscopic examination by a trained operator. Diluted semen is examined at around 100 to 150 times magnification at 37°C. An estimate of the ratio of live to dead sperm is made. An assessment of the relative forward progression (motility) of the live portion is also made. Producers have minimum cut-off points for these parameters, below which the semen is not used.

A trained operator is able to assemble all the information quickly and make a judgement based on experience that enables a decision to be made on semen usage. Within LIC operators use benchmarking and crosschecking to ensure that there is consistency in semen assessments. Operators usually vary by only +/- 5% for the live/dead ratio and +/- one increment (out of a scale 0-10) for forward progression.

It is acknowledged that this visual assessment is subjective. However, within a production system that may have to handle forty ejaculates in a day it is still the quickest method of integrating sufficient data to make a decision that is usable by the producer. Combined with the added acknowledgement that no machine-based system can offer a better correlation with fertility, it is the system of choice for its speed and convenience.

3.17.2 Livestock Improvement's frozen semen quality control tests

LIC's standard in-house test for frozen semen is an incubation of the bulked contents of four straws. Straws are thawed, the

ultrasonically sealed ends cut off and the contents of the straws dispensed into a 3ml agglutination tube. This is then incubated at 37°C for 16 hrs.

A scale of percentage live and forward progression (motility) is used to judge the longevity of the semen. By implication, this is a measure of the damage incurred in the freeze/thaw process; the shorter the lifetime, the more damage has been done. The semen is first evaluated immediately post-thaw. At the end of 16 hours the semen is again examined for percentage live and motility.

A lower cut-off point of 50% live and 6 out of a scale of 10 for motility (or forward progression) post-thaw has been established at LIC.

A further test, based on the above is also used to assess treatments in frozen and liquid semen trials. Semen is thawed, bulked and incubated at 37°C. Semen is examined immediately post-thaw and then again every 24 hours until 10% or fewer sperm are left alive. This is called an ‘end-point’ incubation.

Table 3.3 shows the semen quality control cut-off parameters for frozen semen employed by LIC. Values are for the minimum acceptable standard below which semen is excluded from use. Values are expressed as percentage live (by visual assessment) and a value in the range 0 –10 for motility.

Table 3.3: Livestock Improvement Corporation's quality control minimum values for field usage of frozen semen (supplied by G.Wilson, LIC.)

Semen QC cut-offs	% live	Motility
Pre-freeze evaluation	70	7
Post-thaw evaluation	50	6
16 hour incubation	10	1

3.18 Cold shock

Ejaculated raw (undiluted) semen or semen diluted in non-protective diluent is susceptible to cold-shock. This is a catch-all description of a raft of cellular changes that occur when the temperature is changed rapidly or falls below 5°C for extended periods.

Below 14°C the sperm membrane undergoes a phase change that solidifies it. This stops the action of various cellular pumps, most notably, the sodium pump in the membrane. Sodium levels rapidly build up in the cell and various cellular processes dependent on it slow down and stop. Eventually, non-recoverable, toxic levels are reached and the cell dies unless the temperature is reduced to below zero to halt any further deterioration.

At low temperatures, without the presence of protective agents, the membrane itself is damaged. This damage is also exacerbated by the presence of seminal plasma. It is possible to wash an ejaculate by centrifugation or filtration to dilute the concentration of seminal plasma. However, this will not readily remove peptides already bound to the sperm membrane. Competition for binding sites is a better method for lowering the bound peptide concentration. Egg yolk lipoprotein and some milk proteins that compete for binding sites are the industry standard for sperm protection. Lecithin is also used as it combines with the seminal plasma proteins rendering them less active.

3.18.1 The cold shock test

A simple method developed at LIC for testing protective effects is the cold-shock test. This exposes the sperm to nearly freezing temperatures and manifests as physical damage or loss of viability over subsequent incubation. The standard method for cold-shocking sperm is to quickly lower the temperature to between 0 to 1°C by immersion in an ice-water bath for 30 minutes. Samples are then restored to room temperature before testing.

The effects of cold shock manifest in both gross morphological changes such as returned tails and in subtler effects at the cellular level. These effects are most easily elucidated by incubation. Cold-shocked sperm will have lower survival times and motility levels than unshocked sperm. Cellular studies indicate membrane damage and lowered mitochondrial function. Overall the effect produced is to lower the durability of the sperm and the test is a good indicator of the efficacy of protective agents or the toxicity of additives.

3.19 Industry needs and constraints summarized

3.19.1 Economic need

Selection of superior breeding stock is driven by the economic need for gains in efficiency of conversion of feed into product. Selection criteria for superior productivity traits depend on the farming context in which the animal products are marketed. The generation interval in cattle necessitates long-term strategic planning to anticipate future market demands.

3.19.2 Breeding practices

Cattle breeding for productivity gains can be summarised as the need to maximise the breeding potential of superior parents by enhancing the number of their gametes utilized in the breeding program. In practice this means that the artificial breeding industry has largely concentrated on extending the ejaculate as males produce many millions more gametes than females. The preservation and dissemination of sperm is simpler and cheaper than ova.

In New Zealand the development of liquid semen technology enabling widespread dissemination of superior genes has resulted in large productivity gains in the dairy industry. Alongside liquid semen technology, frozen semen plays an essential part in New

Zealand's dairy breeding program by "filling the gaps" in liquid semen's capabilities. Lowering sperm dose rates in both technologies maximises usage of the very best bulls.

3.19.3 Fertility is essential

The need for fertility is paramount. A non-pregnancy has the double effect of the loss of superior genetic potential and the loss of lactation from the dam. In the New Zealand context in particular this is an intolerable economic blow to the farmer, as he must maintain his herd's lactational cycle in synchrony with the seasonal grass-growth peak in spring.

3.19.4 Industry Standard Packaging

The 135mm x 2.0mm IMV straw configuration is the industry standard package for frozen semen. Sperm recovery from freeze/thawing in these straws is sufficient to maintain an economically viable product. A large, stable, costly infrastructure has evolved around this straw package, which makes it necessary for any new product to use the same delivery device.

3.20 Frozen semen product development

Product development within the frozen semen industry must meet the following criteria:

- ◆ Capability of gains in the dissemination of superior genetics. That is, uses fewer sperm per insemination.
- ◆ Maintenance of high fertility.
- ◆ Use the standard IMV straw configuration.
- ◆ Cost benefits must have a positive return on investment.

The overriding prerequisite is the ability to create more individual semen doses from an ejaculate to further extend the usage of the very best sires in preference to lower ranked bulls. Implied in this is the maintenance of fertility while the sperm dose rate per insemination is lowered.

In the following chapter possible alternative technologies operating within the aforementioned constraints are considered. Mitigation of freeze damage to sperm by various configurations of cryoprotectives and variations of the straw package are discussed. Alternative cryogenic storage systems and temperatures are considered. Within the development sequence of this new product it was deemed necessary to consider all the possible options to the established cryoprotective technology, within the major constraint of retaining the IMV straw.

4 Consideration of alternative technologies

4.1 Introduction

The major constraint placed on the development of new technology for frozen semen is the retention of the IMV straw package. The almost universal uptake of this device precludes adoption of a new package unless great economic benefit is gained. Given that the infrastructural investment around the IMV package is substantial it would take a revolutionary step to change AB practices.

By definition, a change of this magnitude would most likely do away with frozen semen altogether. The IMV package has evolved into the optimal commercial medium for frozen semen. Another technology to package frozen semen is not likely to take over in the market, as the infrastructural investment is so well established that AB companies are loathe to reinvest without a substantial promise of a lift in return on their investment. If a technology were to be developed that stored semen without the need for cryogenics a large portion of the overheads in this system would be removed and costs could be lowered. However, despite many efforts, long-term storage without refrigerants is not a viable alternative. Recent work shows promise but it is still in the early research phase [81], [82].

We are therefore left with the problem of advancing frozen semen technology within the bounds of the IMV straw package. Implied in this statement is the need for each ejaculate to be extended further to optimise the usage of the very elite sires for the reasons explained in Chapter 3 section 3.13.

Lowering sperm dose rates while maintaining fertility is the driver behind frozen semen research. This has almost invariably been approached by attempting to upgrade the performance of the cryoprotective system in the straw [58], [45], [46].

In all current systems the cells are embedded in a homogeneous matrix throughout the semen/extender column in the straw. Protection of the cells is at the membrane and intra-cellular level.

This chapter explores the possible alternative technologies within the above constraints and concludes that no major advancements could be made from further development of the industry standard processes. A new configuration of the contents of the straw that gathered all the benefits of pre-freeze treatment and post-thaw redilution into one package had to be developed. To elucidate this development process it is necessary to understand the effects of freezing fragile cells, the methods of protecting them from damage and the alternatives explored to lower the sperm dose rate.

4.2 Damage processes when freezing cells

Two major damage processes occur when cells are exposed to low temperatures.

4.2.1 Chemical damage.

As the temperature drops, various soluble cellular components of the diluent lose their ability to remain in solution. Also, within the cell and its organelles, areas of differing solubility become more pronounced as the temperature lowers. Osmotic gradients are set up. These can be partitioned by membranes or simply exist in adjacent areas within the cell. As the matrix and the cell move toward the super-cooled state, areas of high solute concentration may remain as super-cooled liquids alongside fragile cellular components such as mitochondria or internal cell membranes. The resultant high osmotic pressures are deleterious to the cell and can cause irreversible breakdown of organelle or gross cell function [83].

The formation of crystals within the cell is also possible. When this occurs within the membrane the result is a loss of integrity that

may not be restored upon thawing. This leads to rapid loss of viability of the cell. Crystal formation within the cytosolic body can be deleterious as organelles are disrupted and internal membranes damaged.

4.2.2 Caprogen contamination effects on post-thaw semen quality

During the early development of Caprogen diluent for ambient temperature semen extension experiments were carried out to determine its efficacy as a freezing medium for sperm. Immediately apparent was a loss of viability post-thaw. Further work determined that some components of the diluent were at their limit of solubility below 5°C and were precipitating in the diluent and within the sperm membrane during the cooling, freezing and thawing phases. This causes irreversible damage to the sperm rendering them inviable in a similar fashion to that described in section 4.2.1.

During the course of this project further work was undertaken to determine the tolerable limit of contamination of the freezing medium with Caprogen. It was necessary to define this limit in order to set tolerances for any possible technology development that attempted to place the optimal post-thaw redilution medium (that is, Caprogen) alongside the freezing medium to allow subsequent rapid redilution.

Semen from six bulls was processed using the RDF protocol developed by Shannon et al [56]. A detailed description of this process is in Chapter 5, section 5.1. Caprogen was added to achieve 5, 10, 20, 30, 40 and 50% contamination in the semen phase. Control semen was frozen using a diluent that omitted the constituents known to cause damage to sperm at low temperatures.

Four straws from each batch were thawed and pooled. 660ul aliquots were diluted into 3.33ml Caprogen to bring the sperm

concentration to 5 million sperm/ml. These were incubated at 37°C to end point (<10% live).

Figure 4.1 below shows the relative performance of the Caprogen contaminated semen.

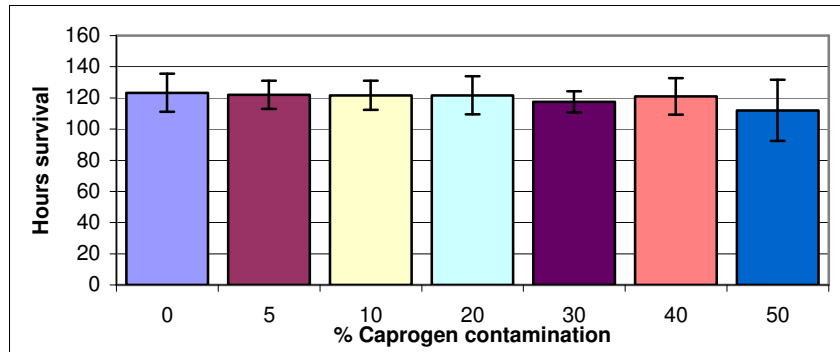


Figure 4.1 Hours of survival of Caprogen contaminated frozen semen

Although there is not a precipitous drop-off in incubation time over all the treatments there is an increase in the variability of the reaction to the contamination at levels above 20%. This is indicated by the larger standard deviations of the incubation times above this level. A decision was made to place a limit of Caprogen contamination to 20% for any further development of technology placing it alongside the freezing medium. This was a conservative choice as bulls' semen varies in its susceptibility to various challenges. This decision was made to exclude any detrimental effects from Caprogen contamination in the most sensitive ejaculates.

4.2.3 Membrane solidification.

Sperm rely on membrane bound pumps to transport metabolites into and out of the cell. The sperm relies on the active transport of sugar from the medium (whether it is seminal plasma or a diluent) to power its metabolic processes. At the same time the sperm moves wastes from its metabolic processes out of the cell into the medium via active pumps in the membrane. At temperatures below

12 – 14°C the membrane solidifies and these pumps cease to work. This can lead to a loss of activity within the cell as substrate becomes limited. Loss of activity *per se* is not necessarily deleterious, as all the cell's processes slow down and produce less wastes and toxic by-products.

However, even at low temperatures above freezing, that is below 14° C and above 0° C there is a slow build-up of low molecular weight metabolic by-products, which cannot be transported out of the cell. Of particular concern is sodium, which is normally actively pumped out of the cell. The membrane pump for sodium shuts down as the membrane solidifies. Eventually cell damage from toxicity will manifest in a loss of the ability to recover viability when re-warmed. LIC includes membrane stabilisers in its Caprogen diluent. The activity of these compounds may be due in part to their ability to lower the melting point of the sperm membrane lipids, thus maintaining the activity of the pumps within the membrane at lower temperatures [14].

4.3 Oxidative damage

In addition to the damage caused by the previously mentioned chemical and physical processes oxidative damage occurs throughout the lifetime of a sperm and an ejaculate, whether it is raw or extended. Sperm produce reactive oxygen species (ROS) that damage other sperm[31], [29], [30], [84]. Dead sperm produce hydrogen peroxide via a reaction involving aromatic amino acid oxidases [24], [85]. This in turn is toxic to other sperm.

Frozen semen is invariably more affected by the production of toxic products from dead sperm as there are more sperm killed in the freezing and thawing processes than in unfrozen systems [86].

4.4 Cryoprotectants

Various researchers have studied the effects of cryoprotective compounds. Their general function is to act as membrane stabilisers and emulsifiers. Many are low molecular weight sugar alcohols that prevent the formation of crystals in the aqueous matrix surrounding the cell. The low molecular weight compound glycerol is membrane transient and will equilibrate within the sperm to protect the cell contents from freeze damage. Glycerol is the industry standard as it has low toxicity and low molecular weight. It is the most successful cryoprotectant for bull semen and when combined with lipoproteins and phospholipids from egg yolk is sufficiently protective to allow recovery from freezing of viable sperm for AI.

Many cryoprotectants are also toxic when applied beyond critical thresholds of concentration and/or time. Semen producers have learned to trade off toxicity effects with the cryoprotective action and apply the cryoprotectants at rates best suited to the semen of the species being preserved. For instance, while the optimum glycerol concentration for bovine semen is 6 – 8 % porcine semen requires 4 - 6% to achieve acceptable fertility.

Glycerol is the mainstay cryoprotectant for cattle AB. Polge et al discovered its effect in the late 1940's and optimal application rates have been established for a variety of buffering systems [42], [43]. Glycerol is membrane transient and mitigates the effects of ice crystal formation within the membrane and within the cell. It has a degree of toxicity and must be introduced into the cell progressively to prevent toxic shock from both osmotic effects and from water withdrawal effects inside the cell. Most dilution systems for cattle semen incorporate an infusion period where glycerol is progressively introduced into the medium and an equilibration period to allow cross-membrane migration into the cell.

4.5 Industry standard cryoprotective system description

The cattle AI industry has developed cryoprotective-buffering systems optimised for freezing bull semen. The most commonly used system has trishydroxymethylamine (Tris)/citric acid as a buffer and egg yolk and glycerol as cryoprotectants. The components of this diluent and their functions are listed in Table 4.1 below.

Table 4.1 Tris cryoprotective diluent components and their functions

Function	Constituent	Action
Buffer	Trishydroxymethylamine/ citric acid	Resists pH change in media from H ⁺ production from metabolic processes.
Cryoprotective	Glycerol	Stabilises membranes & cell contents at low temperature. Emulsifier.
	Whole egg yolk	Stabilises membranes. Competes with toxic seminal plasma proteins on sperm membranes
Metabolic substrate	Fructose	Supplies substrate for metabolic energy needs
Antibiotic system	Penicillin	Bacterial control
	Streptomycin	Bacterial control
Solvent	Water	Carrier

4.5.1 Glycerol alternatives

Dimethylsulfoxide (DMSO), Butanediol and Ethanediol have all been studied as membrane transient cryoprotectants [14]. Their mode of action is similar to glycerol in preventing intra-cellular ice crystal formation thus mitigating damage. Above critical thresholds each can also be toxic to sperm. None has performed as well as glycerol and their higher toxicity has seen them largely discounted as commercial ingredients for bull semen cryopreservation.

Arctic fish proteins (AFP) are produced in fish that live under the ice in the Arctic and Antarctic. These proteins have been the subject of much study as they enable the animals to function at sub-zero temperatures by maintaining cellular water in a liquid state. They have not yet proven

successful in semen cryopreservation although recent work is showing some promise [87].

Trehalose sugar polymer has also been the subject of extensive study as it is produced by desert dwelling plants that undergo repeated diurnal freeze-thaw cycles. Trehalose prevents freeze damage to cells and proteins by modifying ice formation and stabilizing membranes. Although *in vitro* work was promising it has not yet translated into a commercial application. Live offspring have been produced from trehalose treated, lyopreserved sperm but only via assisted reproductive techniques, not from *in vivo* fertilization [88], [82].

Hydroxyethyl Starch (HES) has been used successfully for the cryopreservation of red blood cells [89]. However, no commercial application has been found for HES with semen. Experiments at LIC have showed no positive effects on semen [90].

4.5.2 Egg yolk

Egg yolk (EY) is acknowledged as an integral and essential part of frozen semen technology. EY contains high molecular weight lipoprotein complexes that bind to sperm membranes conferring protection from cold-shock and freeze damage [20; 51].

Research has also been undertaken on fractionating the lipoprotein components of EY to isolate the more beneficial portions [50]. Once again, while there has been promise in the laboratory it does not translate into a benefit in the field. Most production facilities outside Europe use whole EY as a cryoprotectant.

4.5.3 Egg yolk alternative: Lecithin.

Semen producers from the European Union have attempted to create a cryoprotective diluent that excludes EY. As it is an animal derived product EY has the potential to be a vector for several livestock diseases. Frozen semen diluents have been developed that emulate the activity of EY using soya-derived lecithins [91].

Commercial products are available from two European companies (IMV and Minitub) for bovine semen preservation. Commercial semen producers in Europe (most notably, Holland Genetics) have trialed these diluents with varying rates of success [53]. At high sperm dose rates these diluents produce fertility comparable to EY-based cryoprotectants [55], [54]. However, at lower sperm numbers fertility appears to be compromised in these diluents in bulls producing semen sensitive to freeze damage.

4.6 Buffering alternatives.

All successful semen diluent systems incorporate a pH buffer. Changes in pH occur in semen extended for AB as a result of the production of lactic acid via the Krebs cycle in the time pre freezing and post thawing [17]. There are also localised effects as solute and hydrogen ion concentrations change in water pockets left unfrozen as the freezing matrix moves towards the solid state. Solutes migrate into the remaining liquid water and concentrations can rise sharply. Effects are also seen in osmotic pressure and in pH. Consequently buffers are used to modulate these effects [92], [15]. Various organic salt and acid combinations have been tried, along with proprietary biological buffers. This most successful alternative to the Tris/citric acid based system has been sodium citrate/citric acid. This combination does not have the same buffering capacity as Tris but is less toxic. The ability to break down toxic seminal plasma proteins to their constituent monomers is an added benefit of sodium citrate as it renders the monomers of sufficiently low molecular weight to be dialysed away from the medium containing the sperm.

4.7 Alternative freezing technology

4.7.1 Standard freezing protocols

The standard static method of freezing semen at LIC is to place the straws into the vapour phase above liquid nitrogen. The vapour temperature is minus 120°C at a distance of 4cm above the liquid/vapour interface. The

individual straws are held on freezing racks, which hold the straws apart so that the vapour can percolate around them. The straws stay in the vapour phase for nine minutes after which they are plunged into liquid nitrogen to complete the freezing protocol. This regime cools the straws at a rate of around 2.5° C/second. It is largely uncontrolled as there is no feedback mechanism to alter the rate of cooling. Figure 4.2 below shows racks of straws in the supercool vapour phase above the liquid nitrogen which is held at the same level as the grid the racks are standing on. To complete the freeze the straws are plunged into liquid nitrogen through the hole at the bottom right of the picture. Five hundred and fifty straws can be frozen in one nine-minute cycle.

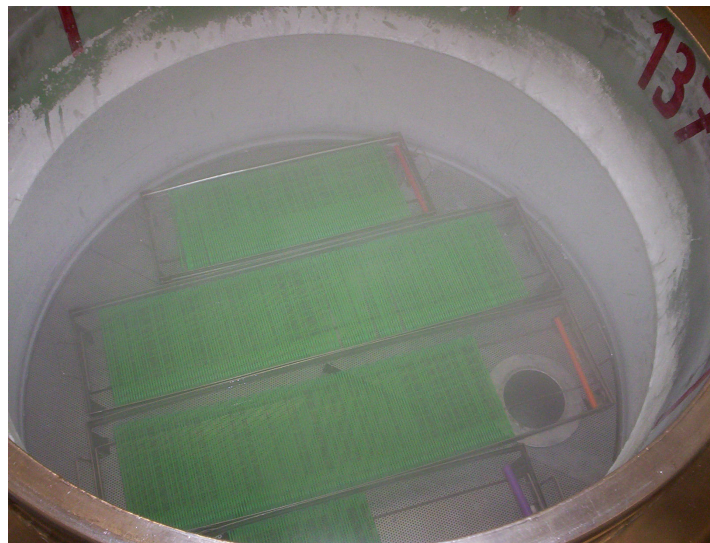


Figure 4.2 Static vapour straw freezing showing racks of straws above the liquid nitrogen level

To standardise and optimise the freezing process, controlled rate freezers have been developed. These use thermostatically controlled feedback mechanisms to introduce supercooled nitrogen vapour into the atmosphere surrounding the racks of straws. Optimal cooling rates for bull semen and other species have been studied and these are applied to the cooling regimes used with these freezers [93]. LIC does not yet use a controlled

rate freezer in its production laboratory. All the semen used in this product development was frozen using the static, passive freezing system in daily use for semen production at LIC.

4.7.2 Freeze seeding

Freeze seeding is the induction of crystallization within the media matrix to speed the transition from supercooled to the solid state. It has been proposed that the frozen matrix formed as a result of controlled seeding is different and more 'glass-like' than an unseeded matrix leading to less membrane damage. The matrix is less ordered (amorphous), with fewer crystalline structures. This lack of order reduces membrane damage and contributes to raise cell survival [94], [95], [96]. One method of physical induction consists of touching the outside of the straw with a supercold wire to create a seeding point. Observations have showed the freezing matrix to be transiting from liquid to solid progressively from the seeding point.

IMV produced a straw with a seeding inducer embedded in the wadding of the plug. Again, their work showed the progression of the transition starting from the plug and moving rapidly through the straw. However, this has not translated into commercial uptake.

Various chemical methods have also been tried to induce seeding. Salts with lower solubility at temperatures near zero precipitate out and create discrete seeding points. While some of these methods showed promise *in vitro*, none has been adopted by commercial enterprises, as no benefit has been shown *in vivo*.

It is taken that the support system used to hold the straws in the cold nitrogen vapour during the freezing process also acts as a seeding point. That is, the racks used to hold the straws often seed the freezing from the points where they contact the straw. Imperfections in the straw, and the plug also can act as seeding points. While these effects have been studied, no conclusive evidence on fertility effects has been shown.

4.8 Physical Packaging Alternatives

While it is acknowledged that the IMV straw package is the industry standard, a paradigm shift could be made if an economic benefit could be derived from a change in the physical storage system for frozen semen. As stated above, the benefit would have to outweigh the cost of a change to a new system. There have been attempts to change the configuration of the packaging for the following reasons:

- ◆ To circumvent existing patent protection. IMV has had intellectual property protection on its straw since the early 1970's. This recently expired which brought at least two more manufacturers into the AB market, CryoVet, and Minitub. Prior to this Minitub was largely unsuccessful in marketing a modified straw using steel beads as sealing devices.
- ◆ To alter heat transfer dynamics. While the straw fulfils all the requirements of packaging, storage and delivery, it is acknowledged that its configuration is a compromise to enable all these functions. Semen recovers better from freezing when it is frozen as a thin film or in a thin flat column. Minitub have produced various straw cross sections to try to take advantage of this.
- ◆ To enable larger volumes to be frozen. LIC has studied freezing in concentrated form (rediluted deep freeze) in volumes up to 20ml using a variety of packages including:
 - ◆ PVC blood platelet bags.
 - ◆ Minitub flat straws.
 - ◆ Kaptan blood bags.
 - ◆ Glass ampoules.
 - ◆ 2, 3 and 5 ml plastic cryovials.
 - ◆ Unpackaged, frozen as droplets on cryogenically cooled aluminium plates.
 - ◆ Frozen in layers in rotating 50ml cryotubes.

- ◆ Frozen as droplets extruded into a 2 metre-cooling tower filled with cryogenic nitrogen vapour to produce a temperature gradient.

The most successful of the above systems was freezing in blood bags. Initially PVC was used but these proved to be fragile at cryogenic temperatures. When Kaptan became available LIC switched over to using custom made bags for its RDF program. The system was field trialed successfully and LIC still has this method available for commercial use [57].

None of the above systems could compete with the IMV straw package for its overall combination of sperm survival and convenient packaging. At the end of the packaging selection process it was acknowledged that any development for frozen semen would have to occur within the constraints of the IMV straw system.

4.9 Alternative Straw Manufacturers

Once it was acknowledged that there would not be a change in packaging type LIC then applied its normal commercial decision making processes to selection of straw manufacturer for supply to its AB operation. In practice, as all the straw manufacturers produce products that perform identically, trial work was unaffected by the source of the straws.

4.10 Alternative Storage Temperature

Alternatives to liquid nitrogen (LN₂) storage were considered to investigate whether this overhead could be lowered. Semen for AB was originally frozen on dry ice and stored in baths of iso-propanol cooled with dry ice. When LN₂ production became economic in the late sixties and the efficiency of storage vessels improved, the industry rapidly changed over to it. LN₂ has a series of advantages over other refrigeration techniques:

- ◆ Cheap, readily available feedstock. LN₂ is made by super compressing air then distilling off the more volatile liquid oxygen.
- ◆ Inert. Nitrogen is almost chemically inert and is completely non-toxic.
- ◆ Tenacious. LN₂ infiltrates all possible niches in a container and is ideal for surrounding objects with a cryogenic medium.
- ◆ Transparent. Objects can be identified through the refrigerant in normal ambient light.
- ◆ Non-residual. LN₂ does not wet objects it contacts. It flows off without leaving a residue.
- ◆ Once produced LN₂ does not require any further inputs to maintain temperature other than efficient insulation. That is, it does not need a power supply and is therefore portable.

LN₂ is in many respects an ideal refrigerant. However, the process of producing it is energy intensive and costly. The vacuum insulated banks used to store LN₂ also represent a capital cost to users.

Electrically powered cryogenic freezers are available that can maintain temperatures below minus 72°C. For variety of reasons these were not seriously considered as viable alternatives to liquid nitrogen for refrigeration:

- ◆ High capital cost
- ◆ High energy consumption
- ◆ Lack of portability

No other refrigeration methods were considered.

4.11 Idea selection summarised

In summary, the following points were the constraints surrounding the product development to address the industry needs and previously outlined:

- ◆ Retained the IMV straw.
- ◆ Frozen in liquid nitrogen.
- ◆ Retained cryoprotectant/s of known, low toxicity.
- ◆ Active reduction of seminal plasma effects.
- ◆ Known buffering system.
- ◆ Retained the standard method of inducing freezing.

This narrowed the field to two candidate technologies described below.

- ◆ Semen dilution into a homogeneous egg yolk/buffering system
- ◆ Modification of an existing method for redilution post-thaw by condensing the system into a single-dose format.

The first option is the obvious choice, as it would restrict any technology changes to within the standard packaging system by simply altering the diluent or pre-freeze sperm treatment. However, lack of progress in frozen semen technologies worldwide excluded this option. Despite the best efforts no major advances have been made for twenty years and it was decided that other options should be explored.

The second option was to take an established technology (rediluted deep frozen) and to repackage it as a single dose.

RDF was well proven in LIC's field system where the semen was thawed and rediluted in the laboratory. Fertility was maintained at sperm dose rates that fell between the liquid and frozen products. The potential gain in production capacity if all the advantages of the RDF technology could be assembled into a single dose package was estimated to be at least 25 percent.

However, placing the frozen semen and the redilution medium alongside each other in a single package had never been seriously considered as an option due to the physical difficulties in maintaining separation of the two media.

Therefore, this project was focused on developing technology to maintain this separation. The following points encompass the beneficial effects that the technique brings to semen extension and preservation:

- ◆ Pre-freeze dialysis to disaggregate and remove toxic seminal plasma proteins.
- ◆ Gradual infusion of glycerol cryoprotectant.
- ◆ Separation from the post-thaw medium at low temperatures to preclude its deleterious effects.
- ◆ Redilution in the beneficial Caprogen medium post-thaw.

Several other material constraints had to be considered. The challenges were manifold:

- ◆ The incompatibility of the post-thaw redilution medium with the frozen semen was the biggest hurdle. A method to maintain separation of two columns of fluid within the same straw had to be developed.
- ◆ Control of the volume of the semen portion. Initial calculations indicated that a semen volume between 15 and 20 *ul* would be needed. To reliably dispense this volume into a straw would require fine control over the metering process. The effects of variability of the volumes on cell numbers in the final product were amplified when using highly concentrated semen samples. Cell concentrations of 400×10^6 sperm per ml were required. At this concentration a one-microlitre error would result in an error of 400,000 cells either as a shortfall or as wastage. Neither scenario was acceptable.
- ◆ Extensive laboratory experience with automatic liquid dispensing systems has showed that microlitre quantities of liquid can be

measured and dispensed reliably. The technical challenge lay in placing the fluid into a specific position within a straw. Automation of the process was also a consideration.

- ◆ Manual production of a working example to demonstrate the theoretical process.
- ◆ It is widely acknowledged within industry that a process and or product that has been manually produced will lend itself more readily to automation. Early manual production of working examples was essential to demonstrate the viability of the concept.
- ◆ The necessity to automate any process developed to meet daily throughput requirements. To produce sufficient quantities of straws embodying the ‘RDF-in-a-straw’ concept for laboratory and field trials would require large inputs of labour, and/or automation. Testing fertility in a statistically robust fashion requires high numbers of inseminations.
- ◆ Maintenance of fertility. Throughout the concept and early development stage the prime consideration was maintenance of semen quality, to ensure fertility was not compromised. The set of tests regularly used and outlined in the previous chapter were applied to all prototypes. The results were so markedly superior to the standard product that the concept rapidly progressed to project status and development was actively encouraged.

The following chapter details the development of the ‘RDF in a straw’ concept from the manual production of prototypes to scale-up of manual production for small-scale field trialing. The design and development of a machine to fully automate the process for extensive field trialing is described. Finally, the conclusion of a national field trial is presented.

5 KNEX Product and Process Description

5.1 Introduction

The Rediluted Deep Frozen (RDF) semen technology was the only known frozen semen system that could achieve acceptable levels of fertility at dose rates below 12.5 million sperm per insemination. However, the RDF product is a liquid at ambient temperature with a limited life-span post-thawing. It then had to be further packaged and transported quickly to its point of use.

A decision was made to try to emulate the physiological advantages garnered by the RDF system into a single dose package that could be kept frozen until thawed at the point of use for insemination. It was hoped that the same lowered sperm dose rate could be maintained, thereby gaining a significant advantage over conventional single dose frozen semen technology.

This chapter describes the product and process development that lead to successful trials of a single dose, frozen semen product that is able to produce 25% more doses per ejaculate than conventional systems.

Livestock Improvement Corporation began a project to develop this product through to 'proof-of-concept'. This was taken to mean a field trial producing statistically significant proof of fertility. The project was given the code-name KNEX.

5.2 Proposed product description

The new "KNEX" product had to fulfil the list of requirements outlined at the conclusion of Chapter four to bring the components of the Rediluted Deep Freeze (RDF) system into a single dose:

- ◆ Pre-freeze treatment
- ◆ Dialysis to disaggregate and remove toxic seminal plasma proteins and the gradual infusion of glycerol cryoprotectant.

- ◆ Separation from the post-thaw medium at low temperatures to preclude its deleterious effects.
- ◆ Post-thaw treatment
- ◆ Redilution in the beneficial Caprogen medium post-thaw.

5.2.1 Pre-freeze semen processing protocol

This was according to the method developed by Shannon et al for the production of rediluted deep frozen semen (RDF) [56]. The steps in the protocol are listed in Table 5.1 below along with an explanation of the function of each part of the process.

Table 5.1: KNEX pre-freeze semen processing protocol

1.	<p>The raw semen is diluted to 400×10^6 sperm/ml in a pre-warmed (32°C) aliquot of non-glycerol diluent containing EY.</p> <p>Introduces EY as a membrane stabiliser and cryoprotectant. Primary semen dilution is near to body temperature to prevent cold-shock. Sodium citrate in the diluent begins to disaggregate toxic seminal plasma proteins.</p>
2.	<p>The diluted semen is progressively cooled to 5°C in a refrigerator.</p> <p>Cooling slows metabolism and production of toxic wastes.</p>
3.	<p>The diluted semen is placed into a dialysis sac (12kda mwco) and dialysed against an adjusted admix of diluents that bring the glycerol content of the overall dialysis system to 8%.</p> <p>Dialysis removes disaggregated seminal plasma proteins and gradually infuses cryoprotectant glycerol to the optimal level of 8%.</p>
4.	<p>After 16 hours dialysis at 5°C the diluted semen is removed from the sac and adjusted to its original pre-dialysis volume with an admix of diluents containing EY and glycerol at the optimal level.</p> <p>Loss of low-molecular weight compounds under dialysis requires adjustment to retain the correct cell concentration.</p>
5.	<p>Semen is now ready for freezing.</p> <p>Semen can be frozen in bulk as in RDF or further processed according to need.</p>

5.2.2 Separation of semen from the post thaw medium

As the lowered solubility of the post-thaw Caprogen diluent constituents at low temperatures was deleterious to sperm survival it was necessary to maintain a physical separation of the semen from the redilution medium during the freezing and thawing processes. This process had to follow a specific sequence of events to arrive at the final product configuration. An understanding of the constraints presented by the straw sealing systems is necessary.

Figure 5.1 below illustrates the unfilled straw and the dimensions of its various functional parts in millimetres.

Considering the general construction of the straw, the following sequence was followed to achieve the desired product configuration:

- ◆ Plug the straw at one end with a porous plug.
- ◆ Inject the components.
- ◆ Seal the straw at both ends.

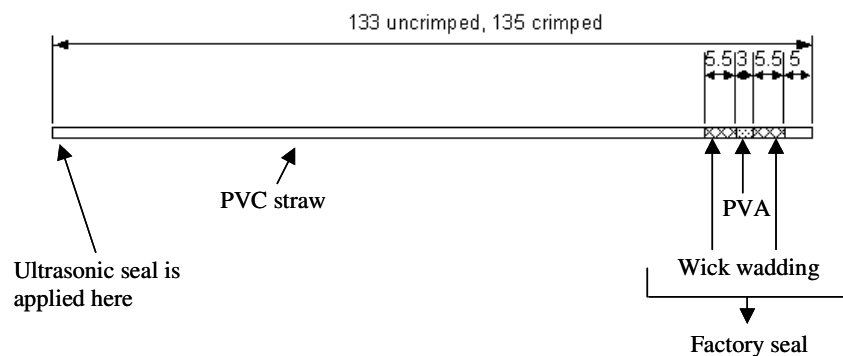


Figure 5.1: Empty PVC straw

The seals are achieved by the following means:

I The porous plug (or factory seal).

Figure 5.2 is a magnification of the porous plug or factory seal showing the PVA powder enclosed at both ends by woven wadding.

The factory seal is attained by wetting of the PVA powder that is contained by two short lengths of woven candlewick within the straw (Figure 5.2). This arrangement is porous when dry. Applying a vacuum at the factory plug end of the straw will generate sufficient negative pressure in the straw to enable liquid to be loaded from the opposite end. The liquid penetrates the innermost wick and contacts and penetrates the PVA powder. The powder then gels and becomes impermeable to further ingress of liquid and to the vacuum.

The factory seal must be wetted sufficiently so that when frozen the aqueous component within the seal is a solid mass that prevents the ingress of any cryogenic refrigerant once the straw has been immersed in liquid nitrogen. Liquid nitrogen within the wadding or PVA powder will vaporize explosively upon thawing of the straw. This will blow the plug out of the straw allowing the thawing water to penetrate the semen dose, or explode the entire package.

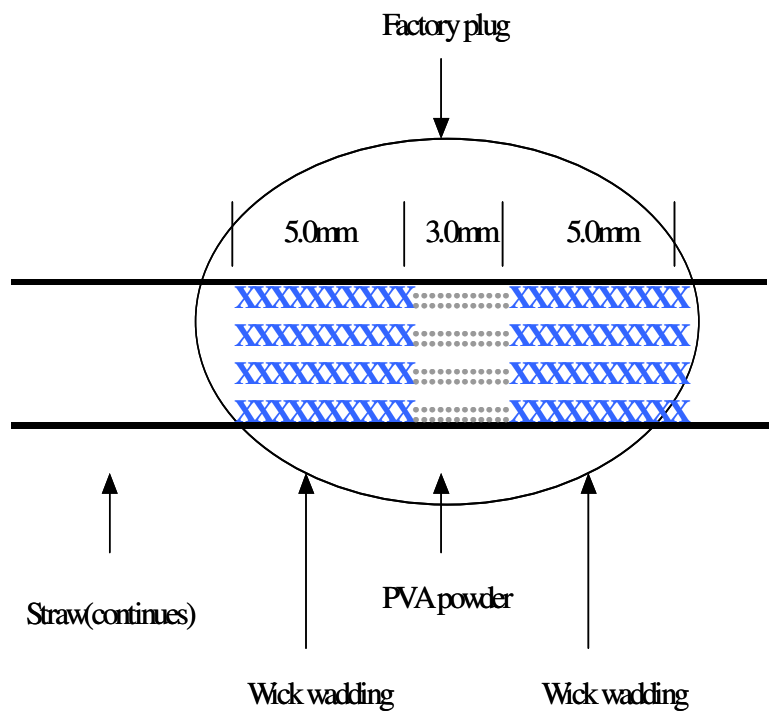


Figure 5.2: Magnification of factory seal. Vacuum is applied at the right hand end. Diluted semen is loaded from the left.

II Ultrasonic seal

The open end of the straw opposite the porous plug is the entry point for the straw contents. Once the straw has been filled it is sealed by ultrasonic crimping and welding. The weld is crimped to 0.2 mm thickness and sealed at 20 kHz.

The ultrasonic seal must be perfect. If liquid nitrogen penetrates the seal and fills the small air space below the seal it will explode upon handling or thawing, destroying the dose. Successful formation of this seal depends on the following:

- ◆ Correct frequency to induce plastic melting.
- ◆ Correct clearances to form the weld.
- ◆ Emitter and anvil shape to contain melted plastic correctly.
- ◆ Weld time.

5.2.3 Injection of straw contents

Diluent was firstly injected into the straw as a separate column. An air gap (gas partition) was then introduced as a physical barrier to contact between the diluent and the semen. A small plug of the dialysed, concentrated semen was then injected into the straw to remain separated from the secondary diluent throughout the freezing and thawing processes.

5.2.4 Redilution in Caprogen post-thaw

This was achieved by shaking down the semen plug into the Caprogen (or secondary diluent) column post-thaw. A simple lab test was developed where the semen plug was replaced with an aliquot of red vegetable dye. Experimentation showed that the dye was distributed throughout the contents of the straw by flicking the straw with the wrist until the air bubble nearest the ultrasonic seal had moved to the other end of the straw and back again.

5.2.5 Semen positioning prerequisites

In the new (KNEX) product the semen would have to be delivered as a discrete plug within the straw, physically separated from the post-thaw redilution medium (Caprogen). The deleterious effects of Caprogen on frozen semen meant that the separation had to be discrete until the components were frozen and also had to remain separate until their temperature was raised above 5°C during the thawing process.

At this point little was known about the stability of the two columns of media within the straw in the pre-freeze state. That is, it was unknown whether the two columns could be maintained separately while the media were still liquid before freezing.

Various options were proposed to maintain separation:

- ◆ A plug of a simplified, non-sperm containing liquid buffer. This method did not work well as the liquid partitioning the semen and diluent allowed diffusion between the two fluids. There was a very short window in which to complete the processing and freezing before contamination of the semen by the diluent became unacceptably high.
- ◆ A semi-solid, injectable plug of a higher density material such as sodium alginate or polyvinyl acetate. This was not pursued as the viscosity of the separating plug made insertion or injection of the separating plug difficult.
- ◆ A mechanically produced crimp in the straw that could be forced open by the inseminator plunger. Crimping the straw without actually sealing it did not prevent the exchange of fluids between the two columns. If a complete seal was formed ultrasonically the inseminator plunger could not go through the straw.
- ◆ A bubble of gas, introduced between the two liquid columns. Experimentation lead to the adoption of this

method as it was the simplest solution and it achieved the objective of physical separation of the liquids throughout the process. Provided the straw was handled gently before the formation of at least one seal, the separation of the fluids could be maintained. Once a seal is formed the configuration was quite robust.

A configuration was settled upon that took into account both the semen positioning requirements and the physical processes required to achieve the final product. Figure 5.3 shows the new component configuration prior to freezing.

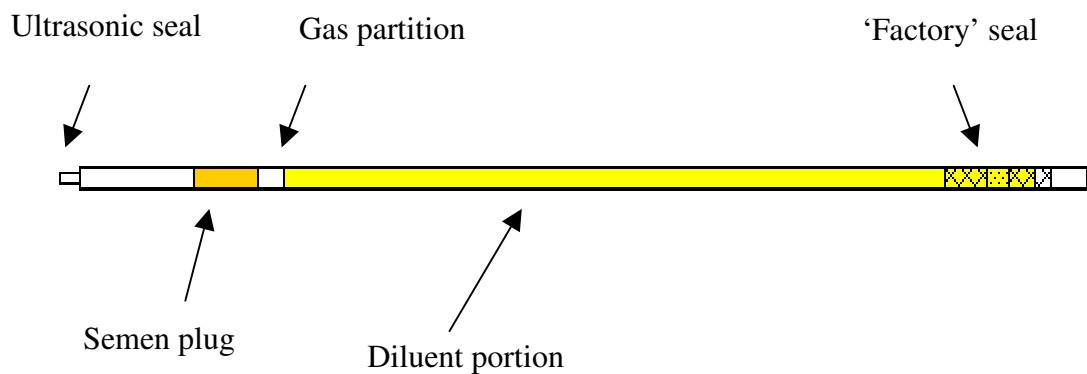


Figure 5.3: Final straw configuration positions before freezing

To achieve this positioning of the components the liquids had to be placed into the straw in a specific order, as shown overleaf in Figure 5.4.

1.

The diluent column was drawn into a point short of the factory plug. This was to allow the subsequent drawing in of the following three components.



Primary diluent position

2.

A gas partition of 5mm of air was drawn in. This action drew the diluent column a further 5mm towards the factory plug.



Secondary diluent position

3.

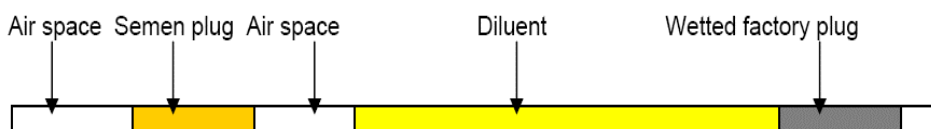
A plug of semen was drawn into the straw.



Primary placement of semen plug

4.

The entire straw contents were drawn across the straw. At this point the factory plug was contacted by the diluent column and activated, rendering it impermeable to further liquid ingress.



Final semen and diluent positions before sealing.

Figure 5.4: KNEX straw filling sequence

Successful trials of RDF commercial product had been frozen at 400×10^6 sperm/ml for redilution to $10 - 20 \times 10^6$ sperm/ml for insemination dose rates of $5 - 10 \times 10^6$ sperm/0.5ml dose. The new product required 7.5×10^6 sperm from a sample of a concentration of 400×10^6 sperm/ml indicating 18.75ul to be placed into the straw. The new single dose system emulated the semen concentration used for RDF, as it was the recognized industry benchmark [56].

The accuracy and precision of the semen volume is critical as sperm numbers in a dose affect fertility. When testing the effects of lowering dose rate, a critical lower limit is reached where any further reduction in sperm numbers will result in a significant drop in fertility. Too few sperm and the fertility drops markedly. Conversely, too many sperm and no advantage is shown as the system is 'flooded' with sperm and the natural maximum level of fertility in the cow population limits the performance. Therefore, there is no gain to the producer. (Refer to: The effects of sperm dose rate on fertility section: 3.2 in Chapter 3.)

Importantly, during the pre-freeze processing, the columns remained discrete and stable to moderate handling once the factory seal had been activated. It was established by trial and error that an air gap of 5mm was sufficient to prevent any capillary action between the two liquid columns. The two columns remained separated in both vertical and horizontal orientation for handling and sealing. The fluid columns expanded into the air gap while freezing. However, the partition re-established after thawing.

5.3 KNEX Product Prototype Production

5.3.1 Manual production of KNEX product for field trialing

Using the standard IMV 0.25ml frozen semen straw, manual filling was achieved by means of a 1ml syringe connected to the straw by a short length of silicone tubing. The syringe was used as

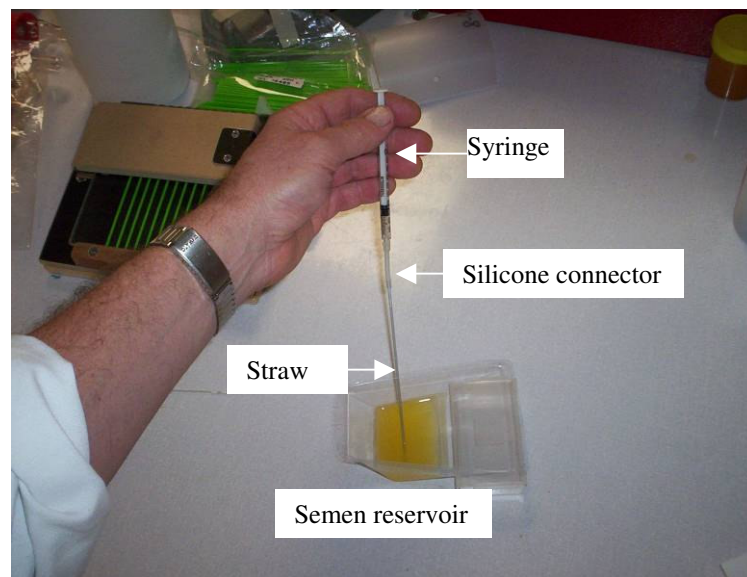


Figure 5.5: Manual filling configuration of straw and syringe

a vacuum pump to create a negative pressure inside the straw to draw in the fluids. Both syringe and straw were held vertically in the operator's hand for insertion into the fluid reservoirs.

In conventional frozen semen systems, with sperm evenly distributed throughout the media, sperm in contact with the factory plug bind irreversibly to the wick component of the plug. An average of 7.5% of the sperm introduced into a straw is lost into the plug. The volumetric loss is 15ul. It was clear that the first liquid to be introduced into the straw in this new configuration would have to be the non-sperm containing diluent to circumvent this loss of sperm. The diluent was introduced into the straw from

a reservoir to a predetermined point in the straw leaving sufficient space to draw in the subsequent components.

Following the diluent, a 5mm air gap was introduced by removing the end of the straw from the diluent reservoir and drawing in the required amount of air.

This was followed by a 9mm column of the pre-processed semen. Finally, these three parts were all drawn across the straw to bring the diluent portion into contact with the factory plug to activate it. This also introduced a second air gap at the opposite end of the straw, drawing the semen away from the open end. This allowed a space in which to form the ultrasonic seal. Once the factory plug had been activated the gelling action of the PVA powder stopped any further movement of the columns. The open end opposite the factory plug was ultrasonically sealed.

The full sequence of events to achieve the required product configuration was shown previously in Figure 5.4.

To ascertain the length of semen column required to equal 19ul of semen, pre-weighed straws were manually loaded and reweighed. This established that a 9mm column contained the required volume. The reproducibility of the syringe loading system was

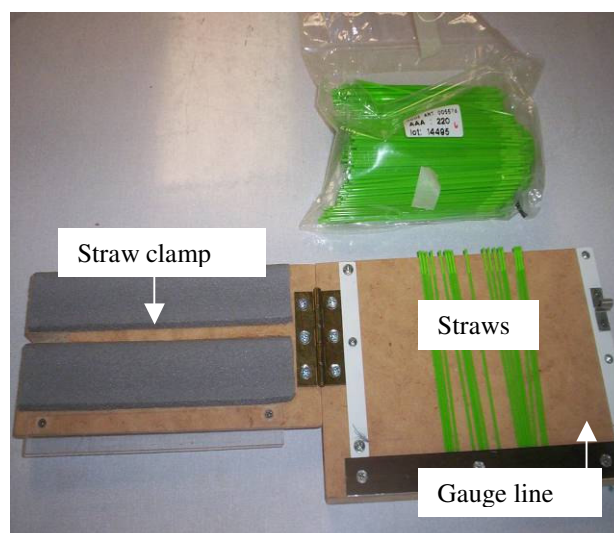


Figure 5.6: Manual straw marking gauge

tested by drawing a line 9 mm from the open end of the straw using a fine tipped marker and a gauge built by LIC maintenance staff (see Figure 5.6). This procedure produced semen columns of length 9mm +/-1mm as measured using an analogue vernier. The gauge allowed consistent marking of multiple straws.

Once it was clear that the configuration of liquids within the straw could be established manually several operators were trained to allow the processing of sufficient numbers of straws to perform laboratory trials.

5.4 In vitro trials of semen quality

The objectives for in vitro trials to prove KNEX product viability were:

Objective 1: To test semen quality, as measured by post-thaw survival at 37°C, which is the standard ‘in-house’ test (described below) employed at LIC to compare KNEX processed semen with the standard product.

Objective 2: To assess the ability to control the loaded volumes of fluids and hence the sperm numbers and semen to diluent ratio in the new product.

5.4.1 Objective 1

I Incubation method to test in vitro post thaw survival

Semen from three bulls of acceptable semen quality (>75% live and minimum of 7/10 motility before freezing) was processed by three treatments:

- ◆ Rediluted Deep Freeze (RDF). Semen was dialysed at 400×10^6 sperm/ml and frozen in single straws.
- ◆ KNEX. Semen from the same dialysis treatment (RDF) above was frozen as a 19ul aliquot in the KNEX configuration in a straw alongside a Caprogen diluent column.

- ◆ Standard Tris based processing system. An aliquot of the same ejaculates used in the treatments above was frozen at 80×10^6 sperm/ml in Tris diluent and frozen in single straws.

The incubation method to test the freezing systems described above was as follows:

- ◆ Treatment 1(RDF): Four straws of the RDF semen (conc = 400×10^6 /ml) were thawed and pooled into a 1.5ml centrifuge tube. A 75ul aliquot was diluted into 925ul of Caprogen in a 3ml glass agglutination tube, which was plugged.
- ◆ Treatment 2 (KNEX): The entire contents of four straws processed in the new configuration were thawed and pooled into a 3ml glass agglutination tube, which was plugged.
- ◆ Treatment 3 (Tris): The entire contents of four straws processed in the standard Tris based system were thawed and pooled into a 3ml glass agglutination tube, which was plugged.

All tubes were incubated in the dark at 37°C until end-point (less than 10% surviving).

5.4.2 Results and discussion

In Figure 5.7 the results of this trial are summarised. RDF processed semen survival time was 87 hours. KNEX processed semen survival time was 80 hours. Semen processed in the Tris system survival was 30 hours.

It was immediately apparent that the new KNEX process produced semen quality and longevity superior to the standard Tris-based process. The benefits conferred by the pre-freeze treatment of the semen and the post-thaw redilution in modified Caprogen were manifest in this single-dose system in comparison to the Tris

system. It was also comparable to the RDF multi-dose system previously used.

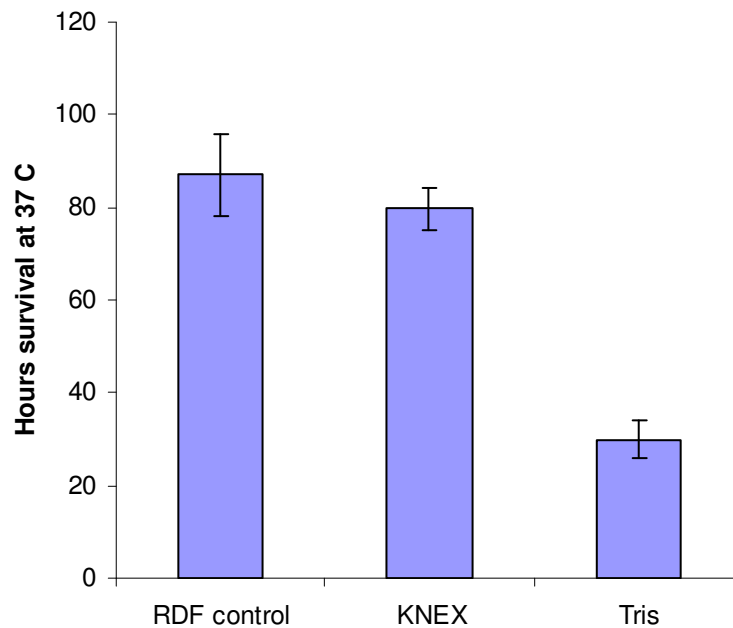


Figure 5.7: Survival hours at 37°C of RDF, KNEX and Tris processed semen.

5.4.3 Objective 2

II Error level assessment of the fluid loading methods

Using an analytical balance for the weight assessments, pre-weighed straws were loaded using the previously described equipment and re-weighed to assess the error in volumes loaded.

III Results and discussion

The manual KNEX process had an error range of +/- 6% or +/- 0.95ul of the semen volume. The error range for diluent volumes was +/- 7%. Experimental design and sample data for these assessments are in appendices 8.1 and 8.2.

Control of the semen volume in the KNEX system was critical to ensure that a fair comparison could be made with conventional systems in fertility studies. As the sperm concentration was very

high in the semen portion of the straw contents it was essential to control this volume to guarantee parity in trials.

The error level for semen volume (+/- 6%) was acceptable for a small-scale field trial as the proposed drop in sperm numbers over the control was to be 40%. The error range of the loading procedure was smaller than the proposed change in sperm numbers. The diluent error level (+/- 7%) was less critical and was also acceptable for a field trial.

At this point the decision to undertake a small-scale field trial was made.

5.5 Field trial 1

5.5.1 Trial objective

The objective of this trial was to determine that the new process had not compromised the semen to the point where no pregnancies were achieved. An unforeseen effect on the semen from the process that completely rendered it infertile would halt the project. This small trial was insurance against a total loss of fertility. Therefore any resulting pregnancies would be deemed a success.

5.5.2 Method

Using the manual filling method described previously, semen was processed from Livestock Improvement Corporation bull code number 98251 who had known fertility. Post-thaw semen quality was assessed and accepted as meeting the LIC minimum standard. (See Chapter 3.17.2 Livestock Improvement's frozen semen quality control tests).

Haemocytometer counts were used to verify sperm numbers in a representative sample of straws from the batch processed.

Forty-two heifers held on LIC's West Block property on Vaile Road, Newstead, Hamilton were oestrus-synchronised using

CIDR-B and prostaglandin according to the protocol developed by MacMillan et al [97]. Oestrus behaviour was observed and recorded in the 24 hours pre and 48 hours post insemination. Oestrus behaviour was also observed on days 20-22 post-insemination to detect animals failing to conceive.

80% of the group showed oestrus clustered around 48 hours from CIDR withdrawal. The heifers were inseminated by two experienced inseminating technicians using standard AI equipment. Straws were thawed, identified and loaded by a technical assistant. Animals were manipulated and observed by experienced stock handlers. Pregnancy diagnoses were performed on day 35 after insemination by a veterinarian using rectal ultrasound examination.

5.5.3 Results and discussion

24% of the heifers inseminated were pregnant. This was sufficient proof that the semen was not undergoing an unseen effect that rendered it completely infertile.

As only forty-two animals were available it was decided to inseminate all the heifers with the experimental semen. It was acknowledged that this design did not include control inseminations from the standard semen processing system. However, the intention was to prove that the semen processing system had not severely compromised fertility rather than to place an absolute value or a value relative to known technology on the NRR of the semen.

5.6 Medium scale field trial

The success of the in vitro trials and the small-scale field trial indicated that the technology would create gains in semen processing capacity without compromising fertility. At this point it was decided that the technology was promising enough to go forward to the next stage. A

decision was made to design a trial in which there would be a comparison with a control treatment using the standard semen process at the commercial sperm dose rate.

5.6.1 Trial objective

The trial was designed to prove equivalence of fertility between KNEX processed semen at 7.5×10^6 SPD and Tris based standard processed semen at 12.5×10^6 SPD at a 95% confidence interval with 90% power.

5.6.2 Method

Ejaculates from five bulls were split into trial and control processes. 1200 trial straws were produced manually at 7.5×10^6 SPD. The control was processed using the standard diluents and equilibration time at 12.5×10^6 SPD. Six representative sample straws from each batch were haemocytometer counted to confirm the processes met the requisite sperm numbers.

The straws were randomly assigned to 41 commercial dairy herds in the Waikato region with the owner's consent. Cows were inseminated as they presented showing oestrus using LIC's standard commercial dairy herd guidelines for insemination. Inseminating technicians were LIC employees with at least one year's previous inseminating experience and known, acceptable ability.

Nine LIC technicians participated and a brief training session was undertaken with each technician. Straws were thawed according to LIC standard procedure of 15 seconds immersion in ambient temperature water. Once the straw was thawed and dried the technician was asked to move the semen column into the diluent column by flicking the straw until the two columns blended. To facilitate further mixing the technicians were then asked to move the air bubble through the liquid column by further flicking of the

straw. The bubble was then moved back to the ultrasonically sealed end of the straw. This was then cut off after loading into the inseminating gun.

A commercial pregnancy diagnosis company (UltraScan Ltd.) was engaged to scan the inseminated animals at day 45-post insemination. Examination was by experienced, trained operators.

5.6.3 Results and discussion

Table 5.2 is a comparison of sperm numbers between treatments as determined by haemocytometer counts.

Table 5.2: Average sperm per dose, n=8. Consecutive batch numbers in adjacent columns indicate one ejaculate split into trial and control treatments.

	Control		Trial	
Bull code	Batch No.	Sperm/dose	Batch No.	Sperm/dose
96329	EP941	15.28	EP942	7.33
96329	EQ153	13.84	EQ154	7.24
98251	EP954	15.44	EP955	5.56
98251	EQ161	12.91	EQ162	7.47
98326	EP881	13.53	EP882	8.28
98326	EQ219	14.13	EQ220	5.82
98476	EP994	17.09	EP995	7.62
98476	EQ143	13.22	EQ144	7.84
98497	EP872	14.91	EP873	7.48
98497	EQ105	12.09	EQ104	7.06

While the best efforts were made to control sperm numbers there was some variation around the objective values of 12.5×10^6 SPD for the controls and 7.5×10^6 SPD for the trial straws. Two trial batches with sperm numbers below 7.0×10^6 sperm per dose were excluded from the results along with their corresponding controls.

The trial semen pregnancy rate was 57.6% compared to control semen at 57.4%. Figure 5.8 below shows the relative performance of the trial semen compared to the control.

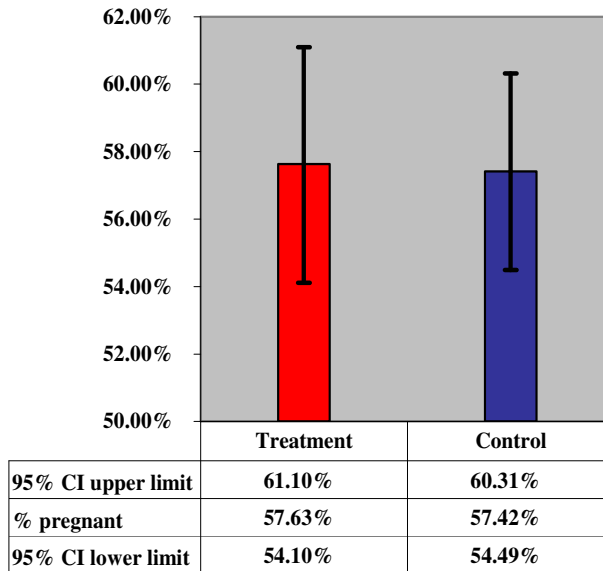


Figure 5.8: Pregnancy rates for KNEX and control semen in field trial 2

Based on this result a decision was made to automate the process to permit larger scale production for further field-testing.

5.7 Automated production of the KNEX product

The next stage in proving the KNEX concept was a large-scale national field trial at a confidence level above 99%. To produce sufficient numbers of trial straws (>20,000) with a suitable level of control over the semen volume (<5% variation) it was necessary to automate the process.

The following performance constraints were imposed:

- ◆ To fill straws with metered volumes of diluent and semen.
- ◆ A suitable air gap between the liquid columns.
- ◆ A suitable air gap from the semen to the end of the straw to allow an ultrasonic seal.

- ◆ No cross-contamination of the two fluids during the filling process.
- ◆ To fill standard frozen-semen straws.
- ◆ The open end of the straws to be ultrasonically sealed.
- ◆ The filled straws should not float in liquid nitrogen.
- ◆ The throughput rate sufficient to meet the trial volumes (approximately 3600 straws/hour).
- ◆ Machine operating temperature of 4°C.

To improve the capability of this prototype machine the ability to vary the fluid and gas volumes being drawn into the straw was introduced as an additional feature.

5.7.1 Straw filling process sequence

Figure 5.9 shows how the straw was filled. The machine had three active processes to add the diluent, the semen, and the air gap at the ultrasonically sealed end. The creation of an air gap between the diluent and the semen was a passive process, created by different length injector nozzles. Standard IMV injector nozzles derived from existing machinery were used. If a variation in the placement or injected volumes was required the length of the injector was altered by means of appropriately sized sealing grommets to change the depth of injector insertion into the straw.

All fluids and gases were loaded from the left-hand side of the diagram. Vacuum was applied from the right-hand side and drawn through the factory plug until its porosity was lost through wetting by the diluent column. As control of the semen volume was critical to the efficiency of the process, semen was metered and injected by a peristaltic pump driven by a stepper motor under software control. The adjustability afforded by this system was essential to fine tuning the semen delivery volume.

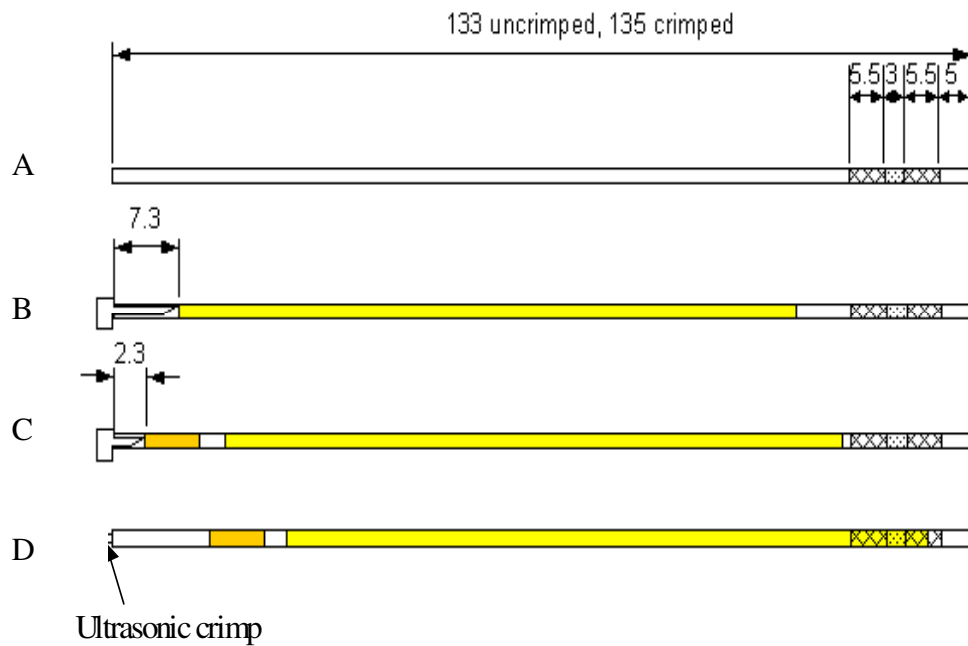


Figure 5.9: Machine filling sequence for fluids and gases

Schematic diagram of filling stages (top to bottom, all dimensions in mm):

(A) Empty straw

(B) Long injector nozzle was used for diluent, which was sucked into the straw by applying suction to the factory seal end. A further constraint on control of the diluent volume was in not wetting the wadding prior to the final air gap being formed. This was essential to maintain wadding porosity to allow further suction for subsequent filling and sealing processes.

(C) A short nozzle connected to the peristaltic pump by silicone tubing was used to inject the semen. This created an air gap between the diluent and the semen.

(D) The final air gap at the end opposite the wadding was generated by suction from the wadding end. This also sealed the straw at the wadding end. The straw was subsequently sealed at the end opposite the wadding using an ultrasonic sealer.

In order to meet the trial objectives regarding control over the injected volumes certain tolerances were set for the product. These are summarised in Table 5.3.

Table 5.3: Allowable tolerances for fluids and gases in the straw

Item	Nominal volume (µl)	Volume tolerance (µl)	Equivalent length (mm)	Length tolerance (mm)	Tolerance as volume fraction (% v/v)
Diluent	180	±5.0	95.2	±2.6	2.7%
Air gap between fluids			5	±1	20%
Semen	19.8	±1.0	10.5	±0.5	5.0%
Air gap to sealed end			15	+0 +2	6.7%

- ◆ The length measurements were based on a straw internal diameter of 1.55 mm, hence a cross-sectional area of 1.89 mm².
- ◆ The tolerances for the two liquids were governed by the volume whereas the air gaps were governed by length. The equivalent length tolerances are shown for the liquids, as that is the simplest way to measure the percentage volume in the straw.

In terms of a percentage the diluent volume had a tighter tolerance although it was more readily achievable as the diluent was introduced at a higher volume. The air gap to the end was specified as a plus-plus tolerance as it was not to be below 15 mm to accommodate the ultrasonic seal. The total air in the straw was not to exceed a volume where the straw would float in liquid nitrogen.

5.7.2 Flexibility of fluid volumes to allow process tuning

As a prototype machine, a level of flexibility was built into the design so that the fluid volumes could be varied and tuned as the

process was developed. The key features of the flexible design were:

- ◆ Adjustable diluent volume.
- ◆ Adjustable air gap between the two liquids by changing the relative length of the injectors.
- ◆ Adjustable semen volume.
- ◆ Capability to adjust the penetration of the diluent column into the wadding seal. This was essential to allow sufficient wetting (by the diluent) to prevent subsequent ingress of liquid nitrogen into the wadding.

5.7.3 Process orientation and conditions

The orientation of the straws in the filling process was to be horizontal. All previous work had used manually filled straws held vertically. The orientation of the filling process was not considered to be a problem. The AB industry had changed from vertical to horizontal filling (of straws) 30 years ago without any problems.

The diluent injection emulated the manual process as it was to be sucked into the straw by the action of a solenoid-actuated syringe pump pulling a slight vacuum in the straw.

The semen was to be injected by a peristaltic pump acting on a short section of silicone tubing.

The entire contents of the straw (diluent, gas partition and semen) were then to be drawn across the straw by the action of a further solenoid-operated vacuum pump to activate gelling in the factory plug.

As sperm are known to be sensitive to shear stresses it was prudent to test the proposed semen pumping configuration in case of any deleterious effects. IRL produced a manual version of the proposed pumping mechanism that consisted of a hand-cranked cam actuating a push-rod impinging on a length of silicone tubing

to displace the liquid in the tubing. *In vitro* testing of semen pumped through this apparatus showed no effect on semen quality. See appendix 8.3 for experimental design and sample data.

5.7.4 Prototype KNEX machine description

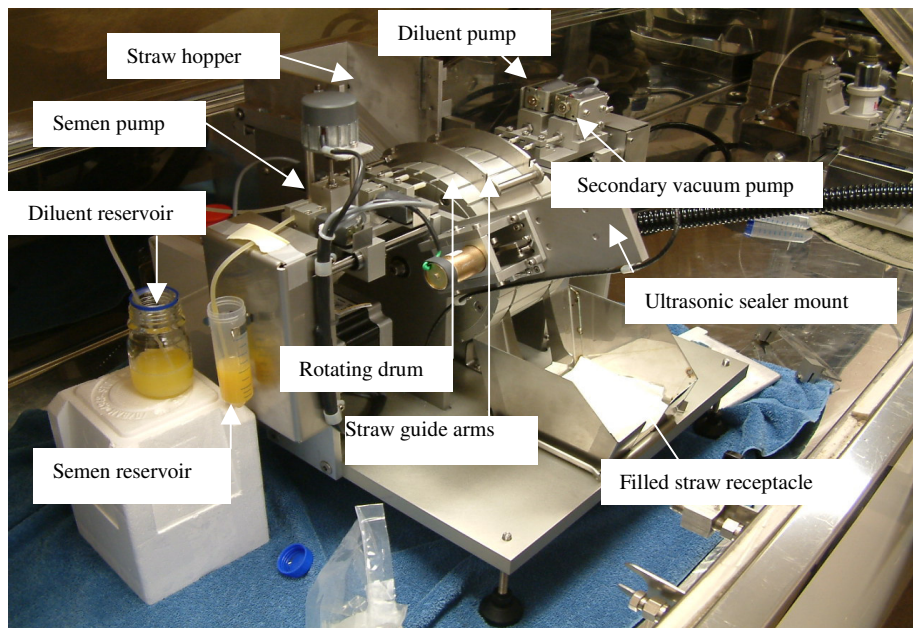


Figure 5.10: KNEX machine general overview in operating mode

A general overview of the machine in operation is shown in Figure 5.10. Fluid reservoirs and lines are on the left of the figure. The straw guide arm mechanism over the rotating drum is an early design that was later replaced by heavier arms following review of their operation. Working drawings and exploded views are in the appendices in Chapter 8.

The working of the machine is best described in terms of the straw transiting the filling and sealing process:

- ◆ Figure 5.11 is a frontal view of the KNEX machine showing straws lying horizontally in the hopper. The factory plug is oriented to the right. Straws are moved to the bottom of the hopper by gravity. At the bottom of the hopper straws encounter a rotating drum that has slots corresponding to the outer dimensions of the straw. The rotational axis of the drum is in parallel with the longitudinal orientation of the straws.

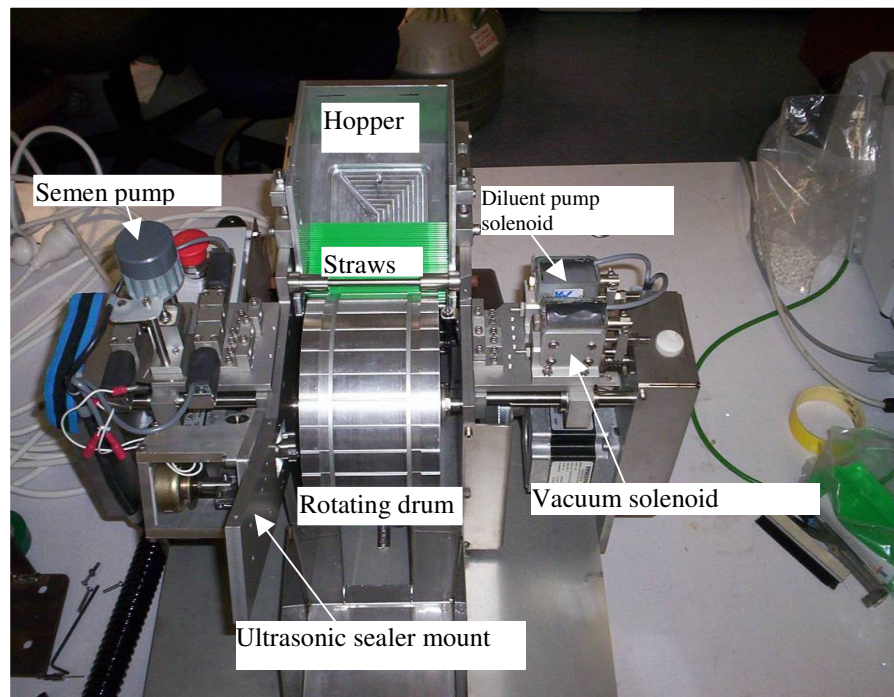


Figure 5.11: KNEX machine general layout frontal view

- ◆ The drum indexes to bring the bottom of the slot in line with the edge of the hopper. A single straw is moved by gravity into the slot and the drum indexes to the next position. On the rotating drum the straw is moved to a vision station where a LED actuated sensor determines the presence or absence of a straw in that slot (see Figure 5.12).
- ◆ Injection station 1; diluent injection: As the drum indexes the straw is moved into line with a vacuum

nozzle at the right hand end and an injector needle at the left hand end. See Figure 5.12.

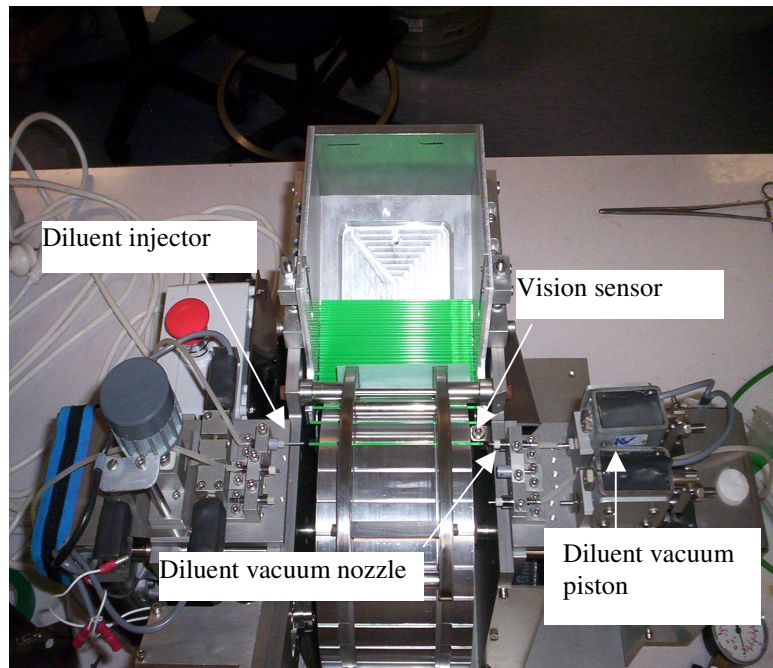


Figure 5.12: KNEX machine diluent injection position, injector carriages withdrawn. Also showing vision sensor location.

- ◆ The injector carriage closes simultaneously with the vacuum carriage, engaging the nozzles at each end of the straw. The diluent vacuum solenoid actuates, inducing suction in the straw by moving the diluent vacuum pump piston. Diluent is drawn up the line from the reservoir and into the straw. The quantity of diluent and the placement in the straw is determined by the stroke of the solenoid-actuated piston, which can be varied. The left hand end of the diluent column is determined by the length of the injector nozzle. See Figure 5.13.

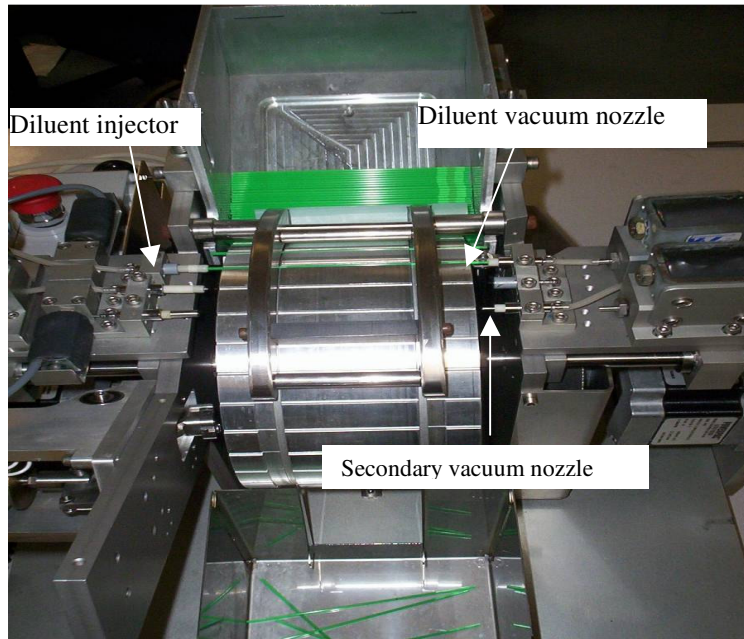


Figure 5.13: KNEX machine diluent injection station showing diluent vacuum nozzle and piston engaged with the straw.

- ◆ The injector carriage and vacuum carriage withdraw and the drum indexes one station to the semen injection station. Both carriages close, engaging the left hand end of the straw with the semen injection nozzle and the right hand end with a buffered, vented stop. The buffered stop is a shortened injector needle holding silicone grommets as buffers. The number of grommets is varied to provide the required length of the stop according to its operational requirements.
- ◆ If the vision system has determined the presence of a straw at the notch adjacent to the semen injector the semen pump actuates to inject semen into the straw. Air displaced by the semen moves the diluent column slightly to the right. Air at the right hand end of the

straw is vented through the factory plug and through the buffered stop. See Figure 5.14.

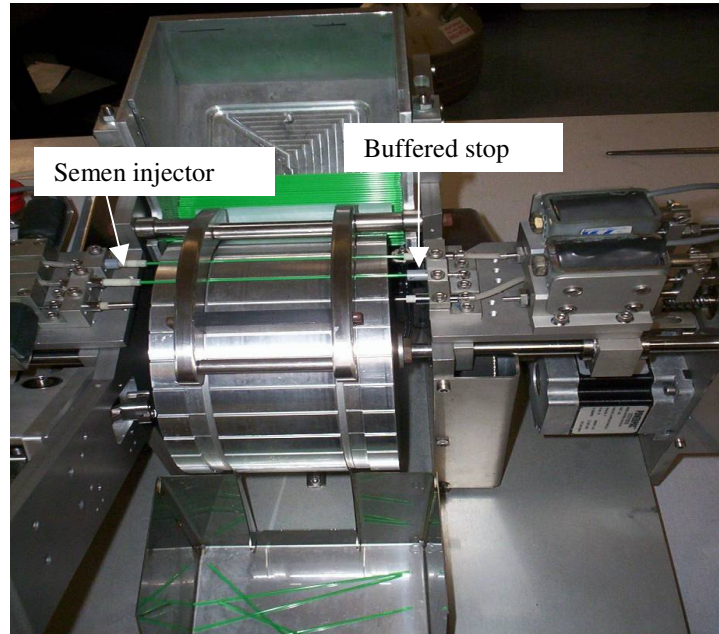


Figure 5.14: Semen injection station, carriages engaged. Buffered stop prevents straw from moving to the right.

- ◆ Both carriages withdraw and the drum indexes one station to the vacuum station. The carriages close, engaging the left hand end of the straw with a buffered stop and the right hand end with the vacuum nozzle. Vacuum is constantly supplied to this nozzle and as a consequence the two fluid columns and the air space between them are drawn across the straw. Once the diluent column penetrates the factory plug it is rendered impermeable to any further vacuum and all movement of fluids stops. See Figure 5.15.
- ◆ Both carriages withdraw and the drum indexes. After four more indices the straw is aligned with the ultrasonic sealing station. The sealing hammer is actuated and the straw is sealed.

- ◆ Subsequent movements of the drum carry the straw to a collection hopper below the drum.

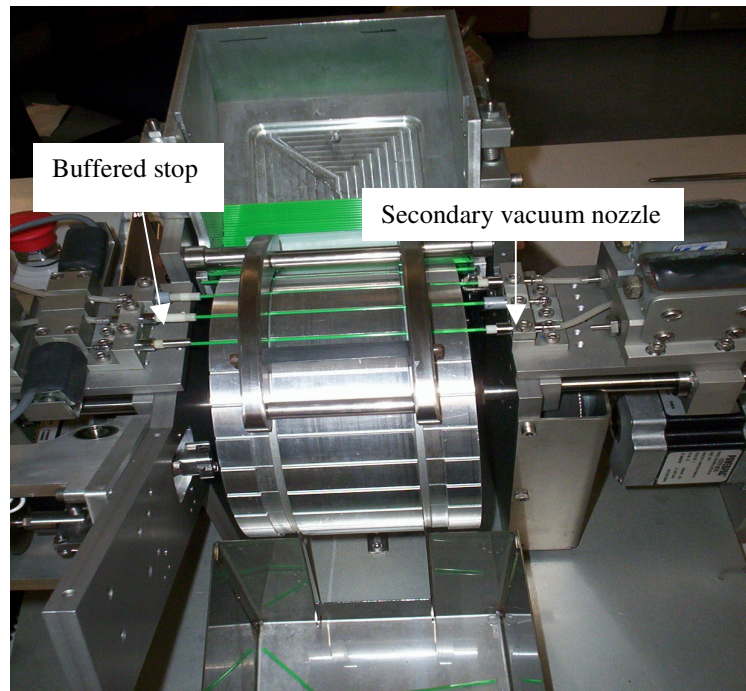


Figure 5.15 Application of vacuum to complete the filling sequence.

5.8 Machine Quality Control

The following control parameters were tested in order to verify machine performance:

- ◆ metering of volumes
- ◆ cross-contamination between the two fluids in the straw
- ◆ throughput

5.8.1 Volumetric Metering Assessment

The determination of the requisite volumes delivered into the straw was carried out by weighing. Determinations were made at ambient temperature (20°C) and at the proposed operating temperature of 4°C unless otherwise stated.

The various filling stages of the machine cycle were interrupted after the point of interest and pre-weighed straws were reweighed

to determine fill volumes. The ability to adjust each of the delivery volumes allowed all requisite volumes for the injected liquids to be met. Experimental design and sample data tables for this assessment are in appendix 8.3.

Determination of gas partition dimensions was by direct measurement in transparent straws using an analogue vernier (Mitsotoya, Japan) and found to be within tolerable limits, (5mm +/- 1mm).

5.8.2 Inter-fluid contamination

As previously stated, the issue of pre-freeze contamination of the semen plug with the post-thaw diluent was of major concern. The components in the Caprogen diluent that were deleterious to the cells during freezing could not be removed from the diluent formula without compromising the performance of the entire package.

It was determined early in the design concept phase that contamination could not be completely eliminated and an acceptable level would be tolerated. This was established in laboratory testing by deliberate contamination over a range to ascertain acceptable cut-off points (see Chapter 4, section 4.2.2).

During early testing it was clear that two possible contamination points in the filling process would need further examination to quantify the contamination level.

The diluent injection nozzle was carrying diluent back to the open end of the straw as it withdrew.

Rapid acceleration of the diluent and semen columns as they were drawn across the straw towards the factory plug was breaking up the diluent column, which then contaminated the semen plug.

5.8.3 Contamination during diluent injection

During testing of the diluent injection process, electrostatic interactions between the filling injector nozzle tip, the straw and the diluent column were indicated by a streak of diluent being drawn off the top of the diluent column. This streak was then drawn towards the open end of the straw, coalescing into a plug.

Experiments were carried out using the KNEX machine to determine the level of cross-contamination between the diluent and the semen plug. Measurement of these volumes was again carried out by weight where it was found to average 23% of the semen plug volume. As this was above the cut-off limit (20% contamination) described previously a delay in the project ensued as a series of possible remedies were tried to reduce this plug:

- ◆ A programmed pulse from the injector nozzle piston after the diluent injection stroke to ‘shoot’ the plug back out of the end of the straw. This was messy and the pulse strength required to remove the plug also shifted another plug off the end of the column to reside at the end of the straw.
- ◆ Construction of a small bar that bent the end of the straw as the drum rotated from the first filling station and suddenly released it to ‘flick’ the plug out of the straw. Once again, this was messy and could not be controlled adequately. The plug was removed some of the time, but at other times the problem was made worse by shifting even more diluent into the end of the straw.
- ◆ Construction of a small platform to hold a section of adsorbent material in contact with the open end of the straw. This was designed to wick away any diluent. While partially successful it did not remove sufficient diluent to be acceptable.

- ◆ The problem was finally solved by having the injector nozzles electrostatically coated with Teflon. It was hoped that the intrinsic hydrophobicity of the Teflon would actively repel the water component of the diluent and maintain it in a contiguous column. The coating was a high wear, low friction formulation (Nilsan 11, applied by Siltech Industries of East Tamaki, Auckland). Tests showed an immediate improvement to an acceptable level.

5.8.4 Contamination during final positioning of liquids

Further testing determined that the next action of the filling process was also causing contamination of the semen plug with Caprogen diluent. Once the diluent and semen liquid columns had been formed in the straw, along with the requisite air gap, the next action was to draw the columns across the straw. This action pulled the diluent into the factory plug and initiated the gelling of the PVA powder. At this point all movement of fluids in the straw stopped, as the plug gelled and became impermeable.

The movement of the columns was by a vacuum induced by a solenoid operated piston pump. The swept volume of the pump could be adjusted by changing the stroke of the piston. This allowed for adjustment of the movement of the columns across the straw and into the factory plug.

The speed of the pump actuation and thus the acceleration and velocity of the fluid columns could not be adjusted. It was an 'all-or-nothing' action whereby the electrical signal to the solenoid was translated into a rapid piston stroke. The movement of the piston did indeed shift the fluids across the straw and into the factory plug. Close examination of the sequence revealed that fragments of the diluent column were being left behind to contaminate the

semen plug as it moved across the straw. An investigation to quantify the level of contamination was undertaken.

Using the KNEX machine individually numbered straws were injected with Caprogen, without semen injection. The straws were then allowed to rotate to the final pull station, the Caprogen was pulled across the straw and the factory plug activated. The straws were then cut at a point above the main diluent column and the piece containing any Caprogen residue was weighed. The cut portion was then rinsed out with a wash bottle to expel the Caprogen, blown out with dry N₂ and dried for 48 hrs at 37°C. It was then re-weighed to obtain the weight difference. Straws were filled at two temperatures; 20°C and 4°C to determine any differences due to filling temperature.

5.9 Machine redesign

The residue of Caprogen left in the semen space averaged 20% of the proposed semen volume when filled at ambient temperature (20°C) and 29% of the proposed semen volume when filled at the normal processing temperature (4°C).

Product throughputs were below 1000 straws/hour in practice. The constant trouble-shooting and adjustments precluded any true assessments of the production rate. It was felt that the throughputs would reach an acceptable level if the problems previously outlined could be addressed.

This delay led to a new phase in the product development. Adjustments and amendments to the automated process were indicated and the machine was returned to the manufacturer for review. While the overall design concept and configuration of the machine was sound, the problems outlined above had to be addressed before the semen product could be properly tested.

5.9.1 Diluent contamination of semen

Two factors contributed to this problem:

I Electrostatic forces

Electrostatic forces between the end of the diluent fill needle and the inner walls of the straw drew semen by capillary action back along the outer surface of the injector needle.

Remedy: This problem was completely overcome by having the diluent and semen fill needles coated with a durable hydrophobic Teflon coating.

II Acceleration of the diluent column

The sudden acceleration of the diluent column at the final pull station left quantities of diluent in the area of the straw subsequently occupied by the semen plug, contaminating the semen.

Remedy: The solution to this problem was to have the column accelerated progressively and drawn across the straw slowly enough to prevent the column from breaking up, while still attaining sufficient velocity to penetrate the factory plug.

The piston block was bypassed and the nozzle was connected directly to an off-board vacuum reservoir via a vacuum regulator. The regulator allowed very fine control over the vacuum level with the consequent ability to fine-tune the acceleration and final velocity of the column within the straw.

Additionally, the action of the vacuum needle connecting to the straw was sufficiently staged to progressively ramp up the vacuum rather than applying it in one instantaneous action. This contributed to the ability to progressively accelerate the diluent column to a velocity capable of penetrating the wadding plug without breaking up the column.

5.9.2 Inconsistent diluent fill

Performance of the diluent injection system was inconsistent. Variations ranged from zero diluent injected to over-filling. The main cause of the problem was thought to be the inconsistent performance of the diluent solenoid/piston block. However, subsequent work remedied this fault without the need for major changes in the injection system.

Remedy: When problems in the straw transport mechanism (see 5.9.4; below), and consequent injector sealing faults were addressed, the inconsistent diluent filling largely disappeared. The piston block was retained.

5.9.3 Solenoid and piston failure

Resetting of the piston at the final pull station sometimes occurred late, after the next straw had engaged with the vacuum nozzle. This ejected the semen plug. The problems outlined previously with the sudden acceleration of the vacuum piston also caused the solenoid and piston to jam at the extreme end of their travel, causing the late resetting of the mechanism.

Remedy: As this mechanism was completely replaced in the fix applied in *II* above this problem was also remedied.

5.9.4 Straw placement problems

It was found that straws did not remain in their correct positions in the slots on the drum. Also, in some instances straws were dawn across the drum, as the injector needles at all three stations on the machine remained connected to them after withdrawal. A further problem occurred at times when two straws would load into one drum slot.

All these problems were traced to the lack of friction between the straw guide arms and the straws on the drum.

Remedy: The straw guide arms that held the straws in place on the rotating drum were replaced with heavier material. The underside of these arms was lined with a strip of urethane foam backed tape. These measures increased the friction on the straws sufficiently to hold them in place.

5.9.5 Hopper chute problems

Straws jammed as they fed down the hopper and did not descend to meet the drum.

Remedy: An oscillating plastic plate was installed in the hopper to perturbate the straws, which were then ordered in parallel for pick-up on the drum slots.

At the completion of this remedial phase and subsequent testing the machine was considered to be capable of processing sufficient numbers of straws with the precision and accuracy required to complete a large-scale field trial. Figure 5.16 shows the revised machine with heavier straw guide arms, a vacuum line to an off-board pump and without the oscillating plastic plate in the hopper.

5.10 National field trial of KNEX processed semen

Following successful shakedown and laboratory trials of the redesigned machine it was decided to field trial the new system in the spring of 2004.

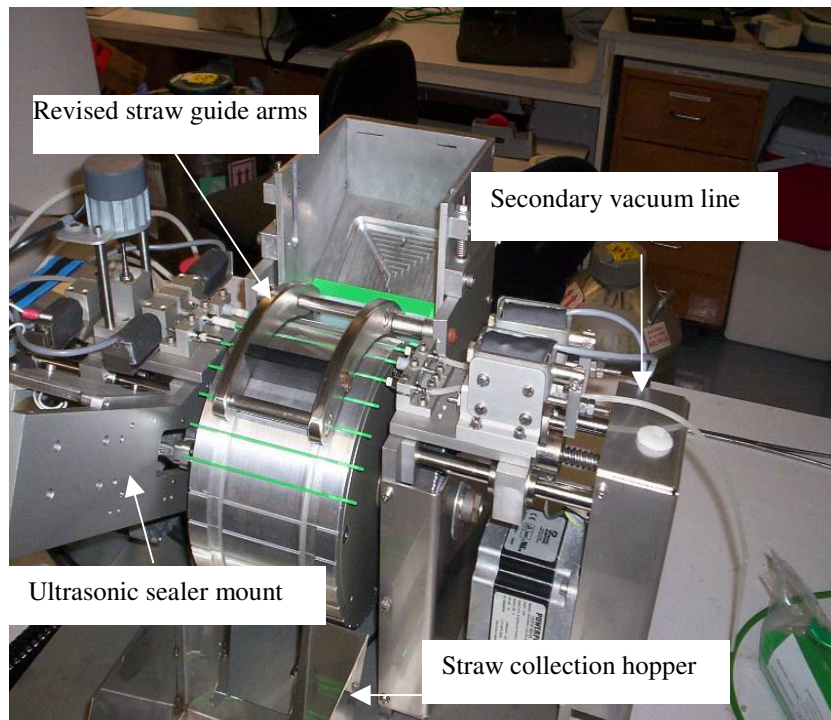


Figure 5.16: Revised machine showing off-board vacuum line bypassing solenoid actuated vacuum pump. Also shows revised straw guide arms

The purpose of a large-scale field trial was to test the fertility of the new (KNEX) semen configuration in comparison to the standard (Tris based) process. The trial design utilised lowered sperm dose rates in comparison to standard commercial practice, (1×10^6 SPD for the trial vs 12.5×10^6 SPD for LIC standard) to model the comparative effects on NRR of the treatment and control processes. The lowered dose rate indicated the need for fewer inseminations. As the lowered dose rate exaggerates any change in semen performance it is possible to use fewer inseminations to show up any difference. This also had the effect of minimizing the risk of reduced fertility, by limiting the number of animals inseminated with trial semen in a herd to three.

5.10.1 Trial Design for National Field Trial

The following points narrowed the potential range of trial designs.

- ◆ Impact on individual customers of possible loss of fertility. This was addressed by placing an upper limit of three on the number of doses used on each farm.
- ◆ Limitations on the machinery capacity. The true capacity of the machine was close to 1000 doses/hour when breakdowns and adjustments were taken into consideration. This placed limits on the number of batches that could be processed within a day, which in turn limited the overall output available to the project.
- ◆ Data collection. As pregnancy diagnosis (PD) is more accurate than non-return-rate (NRR) data fewer inseminations would be needed to gain the same power. Also, inseminations could be spread wider through the mating period, as the data does not rely on observations of return to oestrus. However PD is costly and difficult to coordinate with farmers. A decision was made to rely on NRR as it was felt that sufficient statistical significance could be gained from the quantity of inseminations proposed.
- ◆ To detect a difference of 1% NRR at the standard (12.5×10^6) and proposed new product (7.5×10^6) sperm dose rates would require 100,000 data points in each group (trial and control). As this would entail the processing and despatch of more than 240,000 straws it was clear that there was insufficient capacity within the NZ industry to undertake such a trial.

The reasons for this insufficiency are listed below.

- ◆ Utilisation of straws issued is less than 100%. There would also be losses on distribution due to the variable nature of the make up of technician groups in the lead-up to the mating peak.
- ◆ A proportion of the inseminations would not qualify for inclusion in the results as they would be too late in the farm's mating program for reliable observation of cows returning to service. Generally, once a farm has finished its artificial breeding program animals returning to oestrus are mated by a bull, with little or no accurate recording by farmers of return to service.
- ◆ The risk in exposing the industry to more than 100,000 inseminations from an unproven technology was considered too great.
- ◆ At the lowered sperm dose rate (1×10^6 sperm/dose) the requisite power of the test required 7500 data points in each treatment to achieve 95% reliability. In accounting for losses on distribution and utilisation at least 20,000 straws would have to be processed. It was felt that this would be well within the capability of the new machine to process half this quantity as trial doses.

Straws were processed using split ejaculates from four Holstein-Friesian and two Jersey bulls. The sires were from the upper echelon of the LIC Premier Sires bull team as an incentive to usage; the reasoning was that farmers would be more willing to use semen from the highest genetic merit bulls.

5.10.2 Semen Processing Protocol

A semen processing protocol was followed by which the control process is the standard semen processing procedure used by LIC based on Tris diluent. It is based on a protocol developed by Foote

et al and is used in many commercial artificial breeding companies worldwide [58] (see Chapter 3 Table 3.1 for detailed explanation). The trial process followed the protocol in Table 5.1 developed by Shannon et al for Rediluted Deep Frozen semen [56].

The trial designed relied on the production of trial and control semen at the same sperm dose rates (1×10^6 SPD) in the final product to ensure parity between the NRR calculations.

The following points explain how this was achieved:

- ◆ Raw ejaculate sperm concentration was assessed and the appropriate volume of raw semen was transferred into primary dilutions for both treatment and control for cooling to the processing temperature (4°C).
- ◆ The control semen was diluted to 80% of its final volume and the sperm concentration was recounted. It was then adjusted to the final volume/sperm concentration.
- ◆ Control semen was then packed, frozen and stored.
- ◆ Trial semen was processed according to the protocol in Table 5.1 which was derived from the work of Shannon and MacMillan [56].

The following points should be taken into consideration as they impact on assessment of the relative efficacy of the trial and control protocols and their possible production efficiencies:

- ◆ The average volume delivered from a control straw was 205-210ul. The straw plug uses another 7.5% (i.e. 15ul) but this was accounted for in the trial design by using the delivery volume, not the total.
- ◆ The average volume delivered from a KNEX straw consisted of 150-156ul of diluent plus 18.9-19.1ul of semen.

- ◆ The concentration of sperm in the control straw had to take into account the losses into the plug and the volume delivered and was therefore around 5×10^6 sperm/ml.
- ◆ The concentration of sperm in the KNEX straws had to take into account the small semen plug volume (18.9 ul) required to freeze in comparable fashion to previous KNEX work and was therefore around 58×10^6 sperm/ml.

5.10.3 Field Trial Semen Quality Testing

Two tests were applied to assess the post-thaw semen quality:

- ◆ Standard deep freeze (DF) incubation. Four straws were pooled into a 3ml glass tube for incubation at 37°C.
- ◆ The pooled contents of four straws were rediluted into 4ml Caprogen for incubation at 37°C.

Figure 5.17 below summarizes survival times in both tests on a representative set of samples comparing trial and control batches.

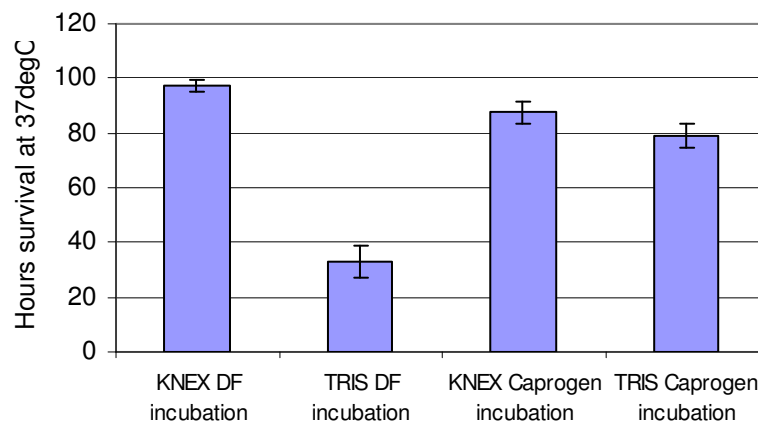


Figure 5.17 Comparison of trial and control survival times. Incubations were undiluted (DF incubation) and diluted in Caprogen post-thaw (Caprogen incubation).

It can be seen that redilution in Caprogen made a small negative difference to the performance of the KNEX processed semen. This

was unexpected as the redilution medium is the same as the diluent portion contained within the straw.

The KNEX and Tris processing systems produced similar survival times when the semen was rediluted post thaw in Caprogen. The KNEX system was shown to be superior when the standard incubation test was applied; that is, without post-thaw redilution into Caprogen.

5.10.4 Distribution of field trial semen

Semen was distributed to inseminating technicians using a model set up by LIC statisticians to ensure that regional and seasonal differences were minimised. Trial and control batches were despatched to adjacent regions so they were used approximately within the same time frame and in areas of similar climate and therefore farming practices. Semen was distributed in late August and early September in readiness for the spring season.

5.10.5 Semen Usage

Semen was distributed to almost all inseminating technicians in LIC employment. To minimise distribution problems technicians were issued either trial or control semen, not both. They were asked to use the straws on the first day of the herd-mating plan. This was to ensure that there was sufficient data return, as most cows inseminated in the first week of mating are observed and recorded for return to service (that is, non-conception).

Not all technicians complied with their instructions. Some semen was not used due to farmers changing their mating plans. A few farmers refused to use the experimental semen.

5.10.6 National Field Trial Results

Table 5.4 below presents the field trial results by bull and batch. The totals and averages are summarised at the bottom of the table.

Consecutive batch numbers in adjacent columns indicate semen processed from one ejaculate split to produce trial and control batches.

Table 5.4 National field trial results by batch summarizing overall NRR comparison between control (Tris) and trial (KNEX) processes. Consecutive batch numbers in adjacent columns indicate semen processed from a split ejaculate

Bull Code	Batch No.	Qualifying	NRR	Batch No.	Qualifying	NRR
	Control(Tris)	insems		Trial(KNEX)	insems	
98251	EY980	683	50.50%	EY981	664	55.00%
99305	FA113	782	51.20%	FA114	732	58.20%
99351	FA221	819	53.10%	FA222	803	56.40%
99416	EY954	728	46.00%	EY955	783	62.50%
99495	EY844	797	53.60%	EY845	779	53.30%
100114	EY898	654	40.10%	EY899	560	43.80%
100114	FA274	823	40.30%	FA275	868	45.90%
	Total	5,286	48.00%	Total	5,189	53.80%

5.10.7 Discussion on trial results

As the semen was used at 1×10^6 SPD the non-return rate shows a difference from the standard picture using frozen semen at 12.5×10^6 SPD. In normal practice a NRR of 66-70% is expected. To amplify any differences between the treatments the semen dose rate was lowered. Despite the dose rate being one twelfth of standard commercial practice the semen still achieved NRR around 50%.

There was a 5.8% difference in NRR in favour of the KNEX treated semen over the Tris control. This was modelled to extrapolate to a potential dose rate difference of 25% fewer sperm required to achieve equivalent fertility with the control treated semen at the commercial dose rate of 12.5×10^6 SPD. That is, the KNEX treated semen at 10×10^6 SPD would have equivalent fertility to the control semen used at 12.5×10^6 SPD.

There is a complex interaction between sperm numbers per inseminate, sperm quality, sperm longevity and fertility levels in the cow. As it is acknowledged that there is an upper limit on fertility on a population basis, the main thrust of this technology development was to lower dose rates per insemination. Implied in this is the knowledge that to detect true differences between treatments the system must be stressed by lowering dose rates to a point below the optimal level where fertility levels off.

The practical implications of the process for a semen producer are:

- ◆ Better usage of elite bulls

The semen dose rate can be lowered by 25% without any impact on fertility. This translates into a higher number of semen doses being available for premium markets where profits are greatest.

- ◆ Genetic gain is enhanced. As the very best bulls are used more extensively genetic gain is faster.

- ◆ Cheaper process

The constituents of the processing media are less expensive than the Tris based system presently in common use.

Labour costs are equal. The same number of ejaculates can be processed without the need for staffing changes. However, more doses are produced.

6 Conclusions and recommendations

The New Zealand Dairy industry is based on the cost-effective management of pasture-fed, fertile cows bred to efficiently convert grass to milk. Fertility is the key driver to farm profitability as it enables synchronicity of lactation with the spring grass-growth peak.

The most important need in the artificial breeding industry is to lower sperm dose rates while maintaining fertility. Additional constraints are maintaining low costs and retaining the standard industry packaging (straws). No long-term, ambient temperature semen storage technology exists and any further development of long-term storage for semen would have to utilise frozen semen technology. However, the problem of high sperm dose rates compared to liquid products has not been solved and the standard Tris-based single dose product has been in use since its development in the 1970's.

In this study, a new product was developed that can overcome some of the deficiencies inherent to existing single dose frozen semen products. It was found that a product contained in the standard industry straw was optimal when sperm and post-thaw diluent was physically separated by an air gap. This prevented sperm from damage caused by the diluent during freezing.

During manual production of prototypes the stability of the two fluid columns and their resistance to cross-contamination was evaluated and found to be acceptable. It was found in early laboratory testing that the process enhanced post-thaw semen survival under incubation by a factor of three over the industry standard (Tris). A subsequent field trial revealed that there was no hidden effect fatally compromising the semen. Equivalent fertility was found in a larger-scale field trial comparing the standard product with the new product containing 40% fewer sperm.

New Zealand farmers have long been supportive of research and development for farming technology. Farmers are quick to adopt new technology and in general willing to participate in trials by offering animals and farming systems for experimentation. The long-term benefits to farmers are borne out in the overall improvement to farming systems in general and specifically to stock, plant cultivars

and animal remedies. Therefore, success in the lab and small-scale trials enabled a large-scale field trial into the feasibility of the new product.

To conclusively prove the efficacy of the new product it was necessary to amplify the difference between the new product and existing products by lowering the sperm dose rates of both treatments to 1×10^6 SPD compared to 12.5×10^6 SPD in LIC's standard. A mathematical model was then used to predict where the fertility became acceptable. Although this pushed the lower limit of fertility, working at higher fertilities would have required a very large sample size, which is impractical and risky for the dairy industry.

The successful field trial of the new product (KNEX) showed that 25% less sperm per insemination can be used compared to the control (Tris) treatment. It was found that with fewer sperm per dose the processing cost is reduced. While the diluent cost for the KNEX product is reduced in comparison to the standard Tris based product, labour cost is unchanged. However, the entire process of collection, dialysis, packaging and freezing fits into a twenty-four hour period to allow a daily production cycle.

Most importantly, the 25% gain in production capacity allows LIC to market more semen from its best bulls. The overall gain in the average genetic merit of the semen offered for sale is a further marketing tool that LIC can use to its advantage.

Despite the success of the new product, the complexity of the manufacturing process and its low production rate lead to a decision to continue research to separately investigate the effect of pre-freeze dialysis and the separate packaging of the semen and post-thaw diluent. This was to determine if adequate benefit could be derived from each process individually. If sufficient benefit could be gained from the pre-freeze treatment alone it would negate the need for the complex and expensive scale-up that would be required to bring the two-part packaging process up to throughput levels sufficient to meet the market demand for frozen semen.

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8 Appendices

8.1 Manual semen filling

Experimental design and example results table to assess variation in the manually filled KNEX straw semen loading volumes.

Semen fill was assessed by drawing a sample of diluent A up to a line drawn 9mm from the open end of the straw, removing the straw from the diluent reservoir and gently tipping the straw into the horizontal position. The straw was then carefully removed from the syringe, wiped and maintained horizontal until re-weighing.

The straws were then wiped and re-weighed.

Table 8.1 Assessment of semen loading error for manual KNEX straw loading.

Straw No.	Straw weight	Weight+semen	Difference	microlitres
1	0.1686	0.1868	0.0182	18.2
2	0.1523	0.1714	0.0191	19.1
3	0.1549	0.1733	0.0184	18.4
4	0.1655	0.1876	0.0221	22.1
5	0.1622	0.1815	0.0193	19.3
6	0.1655	0.185	0.0195	19.5
7	0.1556	0.1761	0.0205	20.5
8	0.1588	0.1787	0.0199	19.9
9	0.1742	0.1942	0.02	20
10	0.1611	0.1812	0.0201	20.1
		Avg	0.01971	19.71
		St.Dev.	0.001114999	
		CV	5.65702057	

8.2 Manual diluent filling

Experimental design and example results table to assess variation in the manually filled KNEX straw diluent loading volumes.

Diluent fill was checked by filling numbered, pre-weighed straws with a syringe to a line drawn 20mm from the plug then removing the straw from the diluent reservoir and then drawing the diluent into the plug to stabilise the column. Straws were then wiped and re-weighed.

Table 8.2 Assessment of diluent loading error for manual KNEX straw loading.

Straw No.	Straw weight	Weight + diluent	Difference	Microlitres
1	0.1588	0.3388	0.18	180
2	0.1625	0.315	0.1525	152.5
3	0.1648	0.3361	0.1713	171.3
4	0.1663	0.3388	0.1725	172.5
5	0.161	0.3362	0.1752	175.2
6	0.1645	0.3521	0.1876	187.6
7	0.1583	0.3554	0.1971	197.1
8	0.1598	0.3455	0.1857	185.7
9	0.164	0.3458	0.1818	181.8
10	0.1605	0.3544	0.1939	193.9
		Avg	0.17976	179.76
		St.Dev.	0.01286582	
		CV	7.157220935	

8.3 Pump configuration

Experimental design and sample results table of determination of the effects on semen quality of various dispensing systems proposed as possible solutions to load the semen plug into the KNEX straw.

- ◆ **A/** Small-scale peristaltic pump dispensing through IMV silicone tube and a machine nozzle; around 60 cycles at 1 cycle/second to dispense 1ml incubation volume.
- ◆ **B/** 60 single dispensings using a mini straw connected via pipette tip to a manual pipettor set on 19ul.
- ◆ **C/** 60 single dispensings using a mini straw connected via pipette tip to a sequential autopipette set on 19ul.
- ◆ **D/** Control. 1ml filled into incubation tube via 1000ul autopipette and blue tip.

All incubations were at 10 million sperm/ml in 5% EY Caprogen at 37°C in 3ml glass agglutination tubes with N₂ atmosphere.

Table 8.3 Assessment of pumping effects of various pumping configurations proposed for the KNEX machine

Bull number	1	2	3	4	5	6	72hr %live	St.dev
A Peristaltic pump	45	60	35	65	55	60	53.33	11.255
B Single dispenser	45	55	45	55	60	50	51.67	6.0553
C Sequential dispenser	50	65	40	65	55	35	51.67	12.517
D Control	40	55	40	55	60	30	50.00	9.3541

8.4 Automated semen filling

Assessment of the semen loading volume error of the KNEX machine.

Straws numbered 1-50 were individually weighed then run through the machine once the semen line had been primed.

No diluent line was run.

Straws were then re-weighed and differences calculated.

Missing values indicate misses by the filling needle. All weights are in μg

Table 8.4 showing sample data is on the following page.

Table 8.4 Sample data from assessment of semen delivery volume from the KNEX machine

Straw No.	Straw weight	+semen weight	semen ul
1	1882	2057	17.5
2	1905	2077	17.2
3	1804	1961	15.7
4	1825	2009	18.4
5	1809	1997	18.8
6	1820	2013	19.3
7	1812	2013	20.1
8	1810	1994	18.4
9	1811	1992	18.1
10	1838	2023	18.5
11	1836	2081	24.5
12	1794	1980	18.6
13	1834	2019	18.5
14	1820	2004	18.4
15	1831	2005	17.4
16	1838	2018	18
17	1782	1959	17.7
18	1830	missed	
19	1802	1985	18.3
20	1813	1996	18.3
21	1820	2000	18
22	1817	2002	18.5
23	1848	2027	17.9
24	1811	1994	18.3
25	1840	2018	17.8
26	1791	1978	18.7
27	1810	1992	18.2
28	1884	2061	17.7
29	1852	2037	18.5
30	1805	1982	17.7
31	1792	1980	18.8
32	1832	2014	18.2
33	1907	2096	18.9
34	1822	2008	18.6
35	1818	2004	18.6
36	1820	2002	18.2
37	1842	missed	
38	1794	1980	18.6
39	1810	1993	18.3
40	1831	2009	17.8
41	1870	2067	19.7
42	1800	1988	18.8
43	1904	2081	17.7
44	1810	1990	18
45	1822	missed	
46	1888	2073	18.5
47	1811	2003	19.2
48	1784	1976	19.2
49	1828	missed	
50	1882	2062	18
		Avg	18.43696
		St.Dev.	1.147046

8.5 Automated diluent filling

Assessment of diluent loading volume error of the KNEX machine.

Straws numbered 1-50 were individually weighed then run through the machine once the diluent line had been primed.

No semen line was run.

Straws were then re-weighed and differences calculated.

Missing values indicate misses by the filling needle. All weights are in *ug*

Table 8.5 showing sample data is on the following page.

Table 8.5 Sample data from assessment of diluent delivery volume from the KNEX machine.

Straw No.	Straw weight	Diluent weight	Diluent ul
1	1594	3387	179.3
2	1627	3510	188.3
3	1597	3436	183.9
4	1621	3430	180.9
5	1614	3464	185
6	1628	3491	186.3
7	1646	missed	
8	1635	3459	182.4
9	1598	3138	154
10	1608	3367	175.9
11	1620	3462	184.2
12	1634	3469	183.5
13	1629	missed	
14	1629	missed	
15	1631	3422	179.1
16	1611	3231	162
17	1644	3497	185.3
18	1621	3415	179.4
19	1637	3481	184.4
20	1628	3394	176.6
21	1610	3404	179.4
22	1642	missed	
23	1596	3385	178.9
24	1660	3524	186.4
25	1626	3490	186.4
26	1613	3330	171.7
27	1631	3484	185.3
28	1642	3228	158.6
29	1618	missed	
30	1620	3518	189.8
31	1638	3424	178.6
32	1616	3445	182.9
33	1628	3443	181.5
34	1601	3417	181.6
35	1615	3430	181.5
36	1623	3494	187.1
37	1632	3441	180.9
38	1635	3212	157.7
39	1611	3278	166.7
40	1649	3481	183.2
41	1649	3462	181.3
42	1638	3499	186.1
43	1628	3223	159.5
44	1650	3494	184.4
45	1616	3471	185.5
46	1646	3450	180.4
47	1628	3453	182.5
48	1619	3479	186
49	1632	3446	181.4
50	1632	3446	181.4
		Avg	179.4933
		St.Dev.	8.652125

8.6 Machine drawings

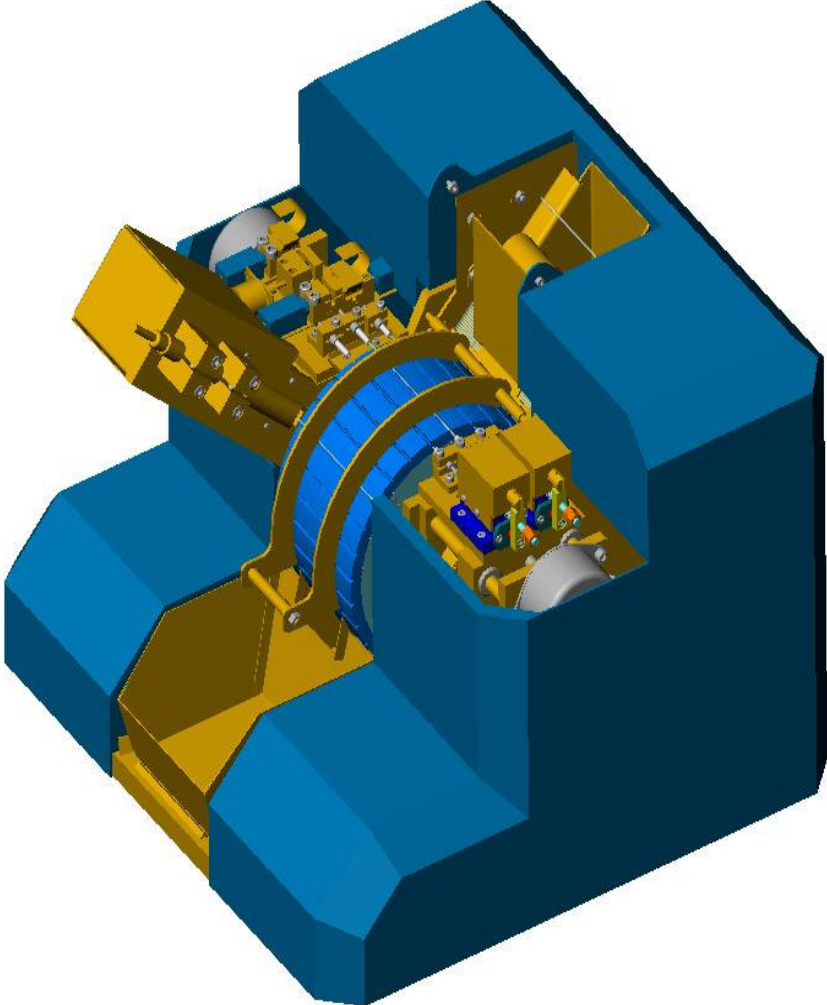


Figure 8.1: KEX machine general overview. Machine is depicted with the covers on, fluid, vacuum and electrical lines disconnected

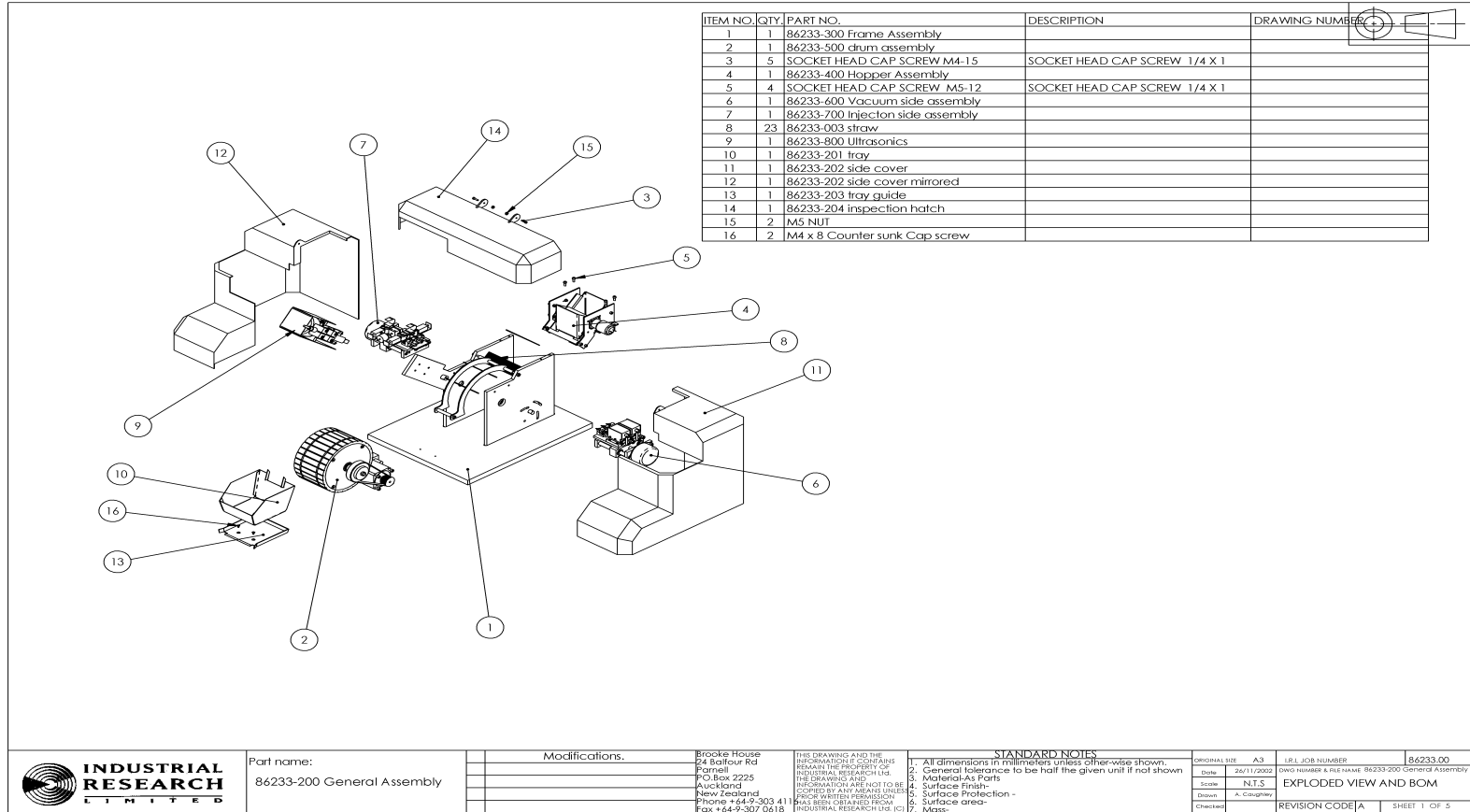


Figure 8.2: KNEX machine, exploded view of hopper, straw transport, injection and vacuum carriages

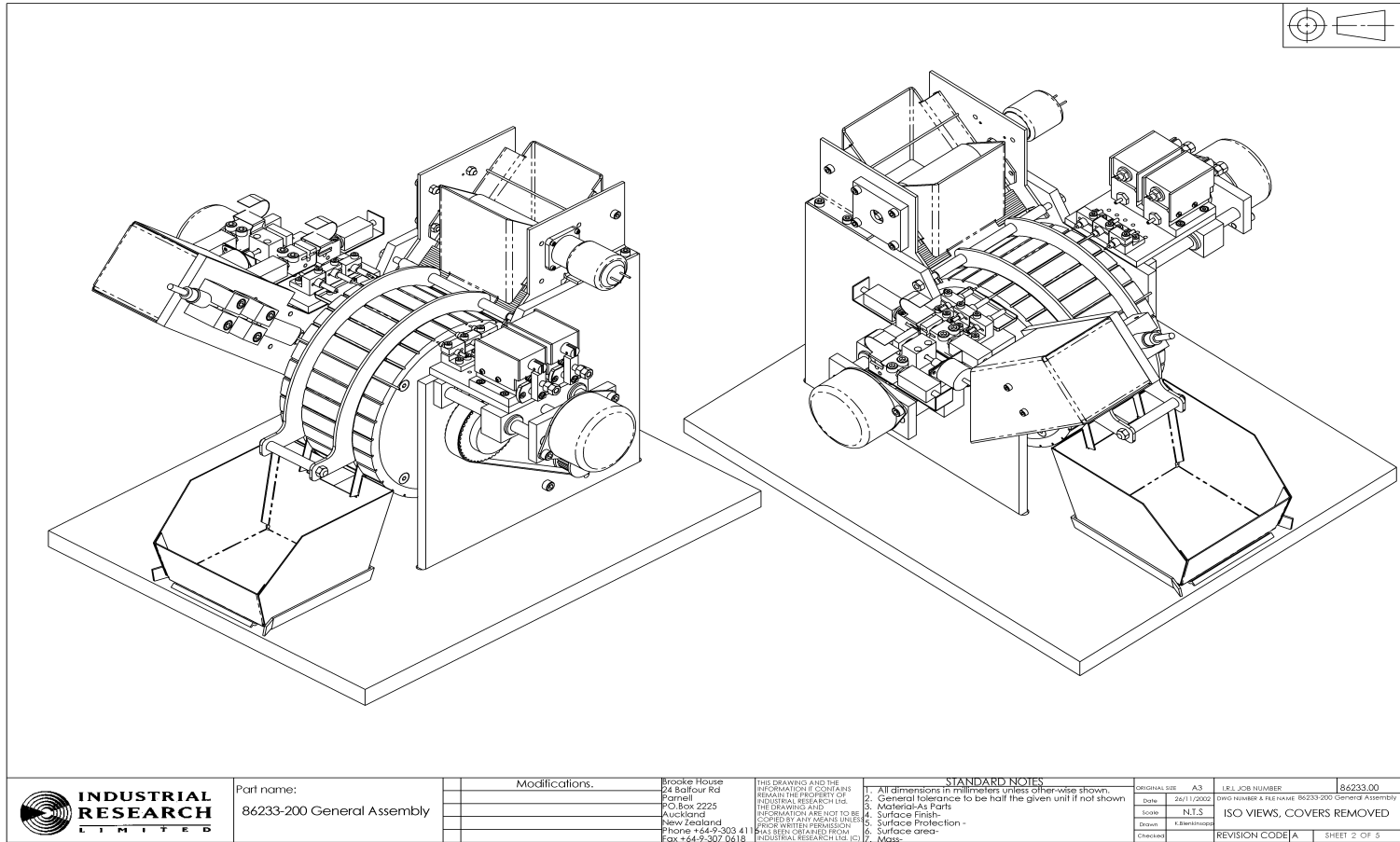


Figure 8.3: KNEX machine general assembly, left and right hand views, covers removed

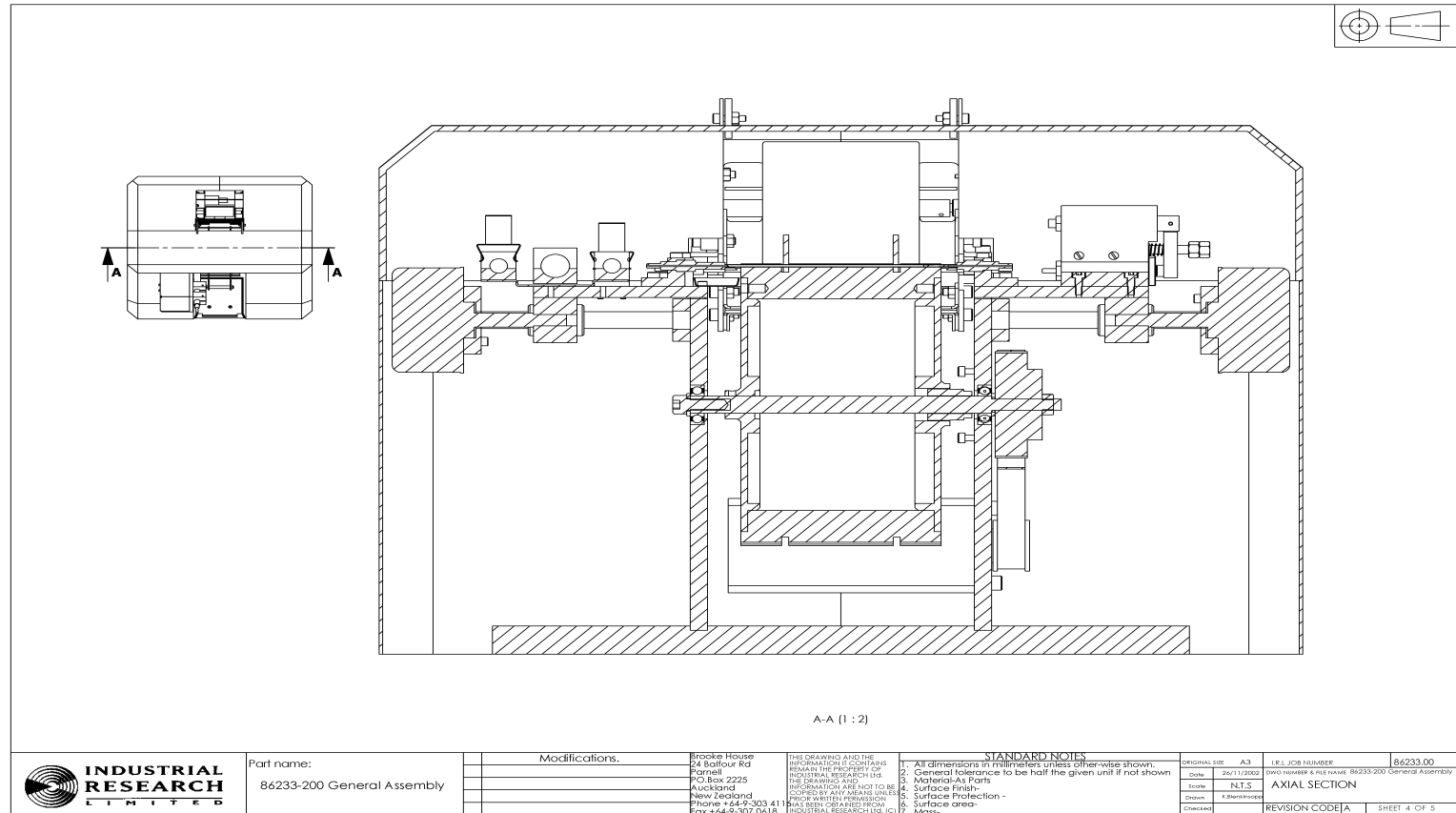


Figure 8.4: KNEX machine, general assembly cross-sectional view through drum.

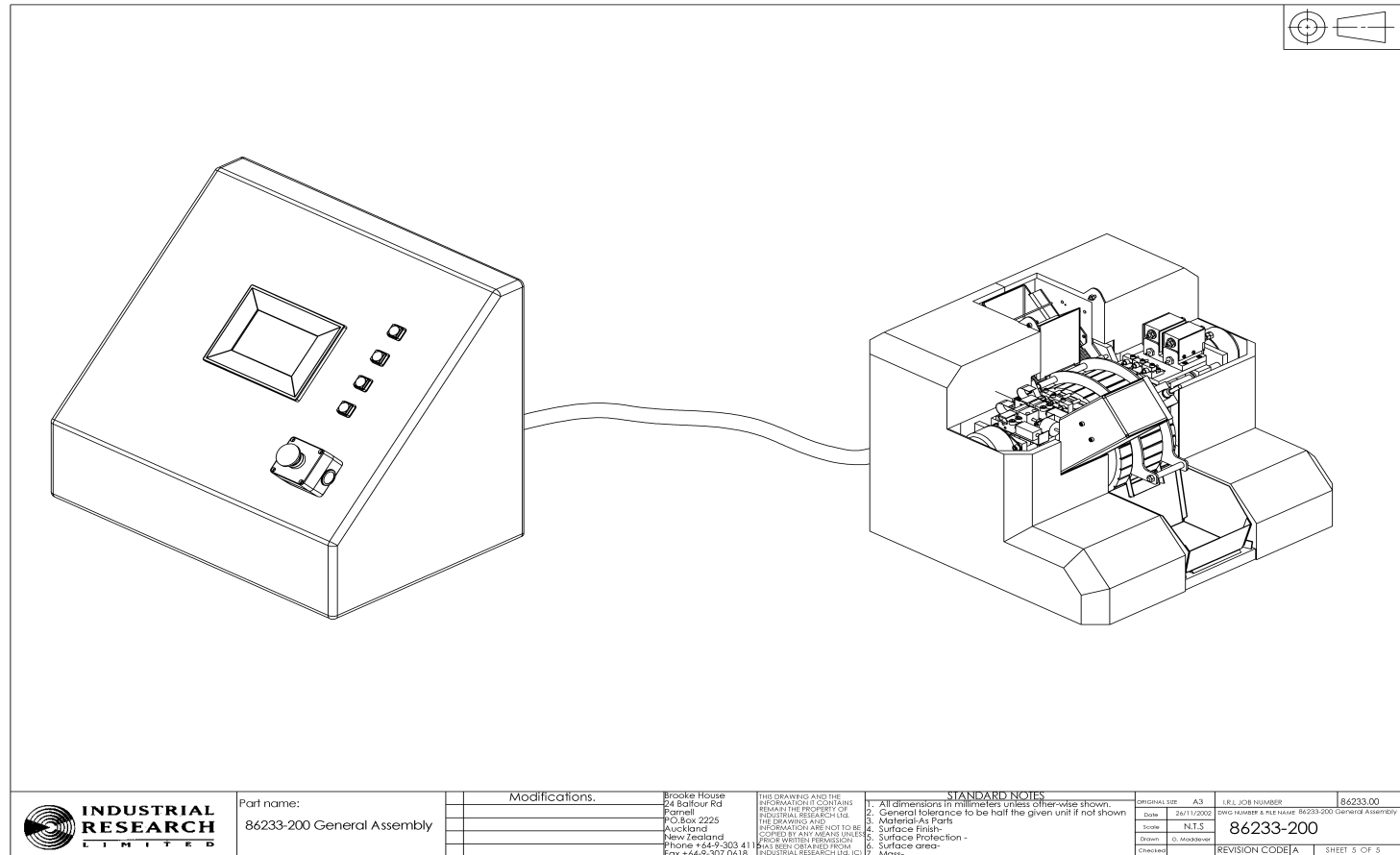


Figure 8.5: KNEX machine general assembly with user interface and control box. Covers on, vacuum and fluid lines disconnected.