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Appendix A:

Risk and Safety Assessment Primer for Animal Cloning

Appendix A:
Risk and Safety Assessment Primer for Animal Cloning

A. How has Risk Assessment Evolved?

Although the overall process of dividing risk assessment into operational steps has been altered to address the nature of the substances or processes being evaluated, the fundamental components of the risk assessment process have remained relatively constant. Thus, for any particular etiologic (causative) agent or process,

(1) the universe of potential outcomes that may be causally associated with exposure are identified and characterized;
(2) the relationships between exposure and outcome are described;
(3) estimates of potential exposure are made; and then,
(4) the qualitative and quantitative (when available) components are integrated into an estimate of the likelihood of the potential outcomes to occur given that exposure also occurs.

Because information for decision-making is often incomplete, risk characterization also must take into account the degree of uncertainty associated with any of the steps in the overall process, as well as the cumulative contribution(s) that such uncertainties may make to the overall risk estimate.

At various times, the National Academy of Sciences (NAS) has attempted to describe risk analysis in different ways (Table A-1). The 1983 NAS report "*Risk Assessment in the Federal Government,"* first attempted to consolidate the risk assessment procedures practiced in the US regulatory agencies (primarily FDA's Bureau of Foods, which subsequently became the Center for Food Safety and Applied Nutrition) into four coherent steps. At that time, these steps were appropriate to the nature of the substances on which risk assessments were performed *e.g.*, radiation and chemical carcinogens.

Chief among the shared characteristics of these substances was the ability to describe dose in discrete units, allowing for the relative precision of exposure and dose-response estimates. By the time of the publication of the NAS's 2002 report "*Animal Biotechnology: Science-Based*

Concerns" (NAS 2002b), the description of the risk assessment process had evolved to be more accurately suited for the potential risks associated with animal biotechnology. The most important differences reflect the change of etiologic agents from radiation and chemicals to biological agents or processes. These differences are most obviously manifested in the hazard assessment and dose-response sections, where the range of potential adverse outcomes (harms) can differ in kind from radiation and chemical damage, and the concept of dose must accommodate biological potential. Biological potential can be thought of as the ability for the substance or organism being evaluated to either grow, replicate, die, or perform a catalytic function so that dose is no longer a constant (or possibly decreasing) amount.

Table A-1: Risk Analysis Steps as Described by the National Academy of Sciences

1983 "Red Book"	**2002 Animal Biotechnology Report**
o Hazard Identification o Exposure Assessment o Dose Response Evaluation o Risk Characterization	o Identify potential harms o Identify potential hazards that might produce those harms o Define what exposure means and the likelihood of exposure o Quantify the likelihood of harm given that exposure has occurred

B. Thinking About Risk

Qualitatively, risk may be thought of as some function of the combination of exposure and the intrinsic properties of the substance or process under consideration by linking an exposure to the likelihood of an outcome. The "risk equation" was first derived for the condition of carcinogen exposure and written as:

Risk = (exposure) x (potency)

where potency was estimated from an evaluation of the relationship between exposure and outcome (*i.e.,* the dose-response evaluation). More generally, however, the risk equation is best thought of as some function of exposure and some function of the biological properties of the agent causing the outcome:

$$\text{Risk} \propto f_{outcome}(\textit{exposure, hazard})$$

In cancer risk assessment, the function of outcomes was often referred to as the "cancer potency" and was derived from the slope of the dose-response curve for tumor formation. For animal

cloning, outcomes may be thought of as the adverse health effects resulting from cloning such as Large Offspring Syndrome, or for edible products of clones, a lack of expected nutritional content of milk from animal clones.

Thinking about risk from the perspective of an "equation" is useful, even when performing qualitative analyses, because it allows the equation to be "solved" for any of the variables that have been defined. Often we ask the "forward" or prospective question: given that some process or exposure has occurred, what is the likelihood of a particular outcome (*e.g.,* how likely is exposure to a particular contaminant in milk to cause gastrointestinal distress?). Alternatively, the question can be asked in the "backwards" or retrograde form: given that an outcome has occurred, what etiologic agent under which exposure conditions is responsible for that outcome (*e.g.,* given gastrointestinal distress, did consumption of milk contaminated with *x* amount of *y* substance cause that effect? or how much of *x* do you have to consume before gastrointestinal distress is experienced?).

When performing a risk analysis, it is critically important to distinguish between a *hazard* and the potential *risk(s)* that may result from exposure. A *hazard* can be defined as an act or phenomenon that has the potential to produce an adverse outcome, injury, or some sort of loss or detriment. These are sometimes referred to as *harms*, and are often identified under laboratory conditions designed to maximize the opportunity to detect adverse outcomes. Thus, such observational summaries are often referred to as "*hazard identification"* or "*hazard characterization."* Risk, as previously discussed, is the conditional probability that estimates the probability of harm given that exposure has occurred. In a qualitative assessment such as this, however, risks can be discussed only within a qualitative context, and no quantitative interpretations should be made.

Another important question to consider is who experiences the risk. At its inception, risk assessment tended to be anthropomorphic; all risks were evaluated in the human sphere, and were expressed in units of the individual, that is, the probability of a person being exposed to a hazard and experiencing a harm over a lifetime. That individual was defined as the *receptor*. Human risks could also be expressed at the population level, or the probability of *x* individuals in the population experiencing the harm. For animal cloning issues, the receptor can be considered to be the surrogate dam carrying a fetal clone, the animal clone itself, or humans or other animals consuming edible products of clones (*e.g.,* milk and meat).

C. How Do We Think About Safety?

For purposes of the Draft Risk Assessment, *safety* may be best thought of as the condition under which risks would be considered unlikely, rather than the condition of no risk (as such conditions do not exist for any scenario). It implies that a risk analysis has been performed, and the "risk equation" is solved for the condition that Risk □ 0 (i.e., the conditions under which risk approaches zero). When considering food from animal clones, this risk assessment has approached the issue of safety from a comparative perspective. Because one of the basic questions that the food consumption portion of this risk assessment asks is whether animal clones are materially different from their conventional counterparts, the risk question that is asked is whether edible products from animal clones or their progeny pose an increased risk relative to the same products from conventional comparators. Likewise, for animal safety, the question that is asked is whether animals involved in the cloning process are at greater risk for any adverse outcome relative to other assisted reproductive technologies.

One of the difficulties with any safety assessment is "proving the negative." Because in practice the universe of conditions under which some risk may be encountered cannot be explored, there are always some conditions under which the null hypothesis (*i.e.,* exposure to *y* µg/liter of Substance *X* will pose no significant risk) will not be disproved. Thus, a careful risk/safety assessment defines the boundaries of its investigation and expresses its conclusions within those particular limits (*i.e.,* clones born after a carefully monitored pregnancy under closely supervised conditions are at a slightly increased risk of dying than animals derived via *in vitro* fertilization, or artificial insemination, or, for food safety, milk from dairy cow clones that meets existing regulatory standards and is not significantly different from Grade A bulk tank milk is as safe to drink as milk meeting existing regulatory standards from Grade A bulk tank milk derived from non-clone dairy cows).

Appendix B:

Overall Reproductive Efficiency and Health Statistics for US Animal Agriculture

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Overall Reproductive Efficiency and Health Statistics for US Animal Agriculture

In order to gain a better understanding of the animal safety issues associated with SCNT, it is helpful to review statistics on animal health and reproduction under current agricultural practices. This section draws data from reports published by USDA/APHIS National Animal Health Monitoring System (NAHMS at http://www.aphis.usda.gov/vs/ceah/ncahs/nahms/index.htm) and the National Agricultural Statistics Service (NASS at http://www.nass.usda.gov/census/census02/volume1/us/index1.htm).

A. Dairy cattle

The Dairy 2007 Part I: Reference of Dairy Health and Management Practices in the United States report (USDA/NAHMS 2007) surveyed a total of 2,194 dairy operations in the United States. According to the USDA/NASS census, in 2002, there were 9,109,600 milking dairy cows in the United States. The predominant breed of dairy cattle in the US is Holstein, comprising 93.4 percent of the national herd. The next most popular breed is the Jersey, comprising about 3.6 percent of dairy cattle in the US. Other "colored" breeds (Guernsey, Brown Swiss, Ayrshire, and others) make up the remaining minority, and numbers for these breeds are more variable. Individual dairies vary in size from fewer than 100 to as many as 10,000 cows. Due to the variability in size of dairies, the USDA/NAHMS report broke dairies down into three groups: fewer than 100 cows, 100-499 cows, and greater than 500 cows. The report does not supply statistics for individual breeds of dairy cattle.

According to the USDA/NAHMS 2007 report, the most commonly reported causes of cow illness for all operations were clinical mastitis, lameness and infertility problems (failure to conceive by 150 days postpartum). Incidence of retained placenta was also a commonly reported problem (7.8 ± 0.2 percent), and may have contributed to incidence of reproductive problems. Incidence of clinical mastitis was similar across operations, and averaged 16.5 percent of all cows. Table B-1 presents data on causes and incidence rate of illness for operations responding to the survey.

Table B-1: Most commonly reported health problems contributing to morbidity, mortality and culling of US dairy cattle

Cause	% morbidity[1]	% mortality[2]	% culled[3]
Clinical mastitis/udder problems	16.5 ± 0.5	16.5 ± 0.7	23.0 ± 0.6
Lameness	14.0 ± 0.4	20.0 ± 0.8	16.0 ± 0.4
Reproductive			
Infertility	12.9 ± 0.3		26.3 ± 0.7[5]
Retained fetal membranes	7.8 ± 0.2		
Other (dystocia, metritis)	4.6 ± 0.3	15.2 ± 0.7[4]	

[1] Expressed as percentage of all cows ± standard deviation of the mean.
[2] Expressed as percentage of cows dying ± standard deviation of the mean.
[3] Expressed as percentage of cows culled ± standard deviation of the mean.
[4] Mortality attributed to dystocia
[5] Culling for all reproductive problems

The percent of dairy cows dying in 2002 was 5.7 ± 0.1 percent, and did not differ by size of operation. The most frequently reported causes of death for all dairy cows in this report were lameness or injury (20.0 ± 0.8 percent), mastitis (16.5 ± 0.7 percent), and difficult labor, also known as dystocia (15.2 ± 0.7 percent).

Mastitis may cause death by acute toxicity, or cows may be euthanized as a result of severe or persistent mastitis caused by treatment-resistant pathogens such as *Staphyloccocus aureus* or *Mycoplasma* species. The percent of cows culled for mastitis or other udder problems in 2007 was 23.0 ± 0.6 percent for all cows culled, and represented one of the most common reasons for culling. Culling due to reproductive problems was an equally common reason given by producers in this report (26.3 ± 0.7 percent of all cows culled), with poor production not due to illness as the next most common reason (16.1 ± 0.7 percent of all cows culled). On average, 23.6 ± 0.3 percent of dairy cows were culled in 2002, with culling rate slightly higher on smaller dairies (24.1 ± 0.6 percent for herds with fewer than 100 cows) compared to medium dairies (23.7 ± 0.5 percent for dairies with 100 to 499 cows) and 23.4 ± 0.7 percent for large operations (more than 500 cows).

Mortality for dairy cattle varied by age, with unweaned heifers having the highest death rate (8.7 ± 0.2) and weaned heifers having the lowest death rate (1.9 ± 0.1 percent). Smaller operations appeared to have a higher death loss among unweaned heifers compared to operations with more than 500 milking cows (9.1 ± 0.4 and 9.4 ± 0.3 percent for operations with less than 100 cows and between 100 and 500 cows, vs. 7.7 ± 0.5 percent for operations with greater than 500 cows).

Table B-2 presents data on causes of death and incidence rate for weaned and unweaned heifers for all operations responding to the survey.

Table B-2: Major causes of mortality for unweaned and weaned dairy replacement heifers that died

Cause	Unweaned	Weaned
Diarrhea	56.5 ± 1.3[1]	12.6 ± 1.0
Respiratory	22.5 ± 0.9	46.5 ± 1.7
Dystocia	5.3 ± 0.7	NA

[1] Percentage of deaths ± standard deviation of the mean.

In the US, most dairy cattle are bred by AI, although many dairies still maintain bulls for cows that do not conceive to AI. According to the USDA/NAHMS 2007 report, 51.7 percent of dairies surveyed maintained one or more bulls. Embryo transfer has been promoted as a commercially feasible assisted reproductive technology (ART) for dairy cattle, particularly for dairies interested in using their best cows to improve herd genetics (Webb and Drost 1992). Embryos are also sold nationally and internationally to increase genetic advancement and overall herd production. The International Embryo Transfer Society, a professional society whose membership includes breeders and researchers, estimates that a total of approximately 670,711 *in vivo* derived bovine embryos were transferred worldwide in 2006 (Thibier 2007). Cows with less desirable genetics or production levels may be used as recipients of higher genetic merit embryos. However, ET is not a predominant means of reproduction in dairy cattle. *In vitro* fertilization has been less successful than *in vivo* fertilized ET, and is not commonly practiced. The developmental competence of cultured bovine embryos remains low (Betts and King 2001), with less than half of bovine IVF embryos developing to blastocysts, and even fewer survive to attachment in the uterus.

In cows bred by AI, pregnancy may be diagnosed by ultrasound 35 days after insemination or by palpation approximately 40 to 45 days after insemination. Average pregnancy loss following a positive pregnancy diagnosis for all cows across operations of different sizes was 4.5 ± 0.2 percent. Pregnancy loss was highest on larger operations (5.3 ± 0.3 percent for operations with greater than 500 head; 3.7 ± 0.1 percent for operations with less than 100 head; 3.7 ± 0.3 percent for operations with 100 to 499 head) (USDA/NAHMS 2007).

B. Beef Cattle

Beef cattle in the US are managed under various systems, depending on the intended use of the animals. Beef cattle destined for slaughter may change hands several times before final disposition. Breeding stock and young nursing animals may be maintained on range or in pasture. These are generally referred to as "cow-calf" operations. Following weaning, animals destined for slaughter may go directly to feedlots or may be maintained for a brief period on high quality pasture, a stage referred to as "back-grounding" or "stocker." In the US, most cattle are slaughtered between 15 to 18 months of age.

The 1997 Beef Cow-Calf Health and Health Management Practices report (USDA/ NAHMS) surveyed 2,713 beef cow-calf operations throughout the United States, representing an estimated 34,280,000 head of cattle. According to the survey, approximately 1.5 ± 0.1 percent of breeding cattle, including weaned replacement heifers, cows and bulls, died or were euthanized due to various causes in the previous year. Mortality rate was higher on small operations with less than 50 cattle, compared with larger herds (2.4 ± 0.3 percent). Approximately 20 percent of these losses were due to unknown causes. The largest single category (27 percent) of losses for beef breeding cattle was "other known" causes, most of which producers attributed to old age. The next highest categories (after "unknown") were weather (18.0 percent) and calving problems (17.0 percent). Table B-3 presents the leading known causes of death, where a specific cause was named, for cattle that died.

Table B-3: Causes of death for beef breeding cattle (cows, bulls and weaned replacement heifers) that died

Cause	Percent ± SE
Digestive	6.1 ± 0.1
Respiratory	6.0 ± 1.0
Weather	18.0 ±3.9
Dystocia	17.0 ± 1.9

Relatively few breeding females in cow-calf herds experienced health problems, according to the 1997 survey. In general, replacement heifers experienced a higher percentage of illnesses compared to mature cows. Pinkeye was the most commonly reported illness, and occurred in 1.3 percent of female breeding cattle. With the exception of pinkeye, illness rates for breeding females appeared fairly similar among herds of different sizes. Pinkeye incidence was reported highest in small herds (less than 50 head, 2.3 percent) than in large herds (more than 300 head 0.6 percent). There was no difference in incidence rate of retained placenta or uterine infections between small and larger operations (0.2 ± 0.0 percent for operations with less than 50 or more

than 300 head). Incidence of pregnancy loss was also small and not significantly different between breeding females in different sized herds (0.2 ± 0.1 percent in herds with less than 50 head; 0.3 ± 0.0 percent in herds with greater than 300 head). Major causes of health problems in breeding female beef cattle are listed in Table B-4.

Table B-4: Causes of morbidity in breeding female beef cattle[1]

Conditions	Replacement Heifers	Cows	All Females
Respiratory Disease	0.9 ± 0.3[2]	0.3 ± 0.0	0.4 ± 0.1
Scours	1.0 ± 0.2	0.4 ± 0.1	0.5 ± 0.1
Pinkeye	1.9 ± 0.4	1.2 ± 0.1	1.3 ± 0.1
Cancer eye	0.0 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
Foot rot	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
Mastitis	N/A	0.2 ± 0.0	0.2 ± 0.0
Retained placenta/metritis	N/A	0.4 ± 0.0	0.3 ± 0.0
Spontaneous abortion	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
Neurologic problems	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

[1] Expressed as average percentage of all breeding females in 1996 cattle inventory.
[2] Percentage of females by category ± SE.

Average mortality rate of unweaned calves was approximately 3.4 ± 0.1 percent of all calves born during 1996, and there were no appreciable differences among operations of different sizes for calf mortality. Two of the most common causes of death, when death could be attributed to a cause, were respiratory problems and dystocia. The leading causes of calf mortality according to producers surveyed are expressed in Table B-5.

Table B-5: Most common perceived causes of death for unweaned calves

Cause	Percent ± SE
Digestive	14.4 ± 1.0
Respiratory	16.3 ± 1.2
Weather	20.2 ± 1.4
Dystocia	13.9 ± 1.3
Unknown	17.5 ± 1.4

The leading cause of morbidity in calves was scours (diarrhea) affecting 2.4 ± 0.2 percent of all calves three weeks old or younger. Older, but still unweaned calves had a slightly lower

incidence of scours (1.7 ± 0.2 percent). Diarrhea, in part, may have contributed to death losses due to digestive problems. Causes of morbidity in unweaned calves are listed in Table B-6.

Table B-6: Causes of morbidity in unweaned calves[1]

Cause	3 Weeks or Less	Over 3 Weeks Old
Respiratory	0.5 ± 0.1[2]	0.8 ± 0.1
Scours	2.4 ± 0.2	1.7 ± 0.2
Pinkeye	0.1 ± 0.0	1.1 ± 0.1
Foot rot	N/A	0.2 ± 0.0

[1] Based on all calves in survey.
[2] Mean Percentage ± standard error.

C. Swine

The total US swine population was estimated at 59,848,000 head in 2000 (USDA/NAHMS 2001). Most US swine operations are fully integrated. This means that swine remain on the same operation under the same general management throughout their lives. Animals are usually maintained under full confinement in highly biosecure facilities, to minimize disease transmission and for other economic and management reasons. Sows generally farrow (give birth) twice a year. Piglets remain with their dams for approximately 21 days, and then are weaned and moved to a nursery, where they are housed in small groups in raised pens for 6 to 8 weeks. They progress through "grower" and "finisher" phases, depending on weight, and are generally maintained in the same groups throughout the process.

The most complete survey of swine health and management practices in the United States was published in 2001. This section derived data from Part I: Reference of Swine Health and Management in the United States, 2000 and Part II: Reference of Swine Health and Health Management in the United States, 2000 (USDA/NAHMS 2001). A total of 2499 producers were surveyed for the report. In order to qualify for the report, operations must have had at least 100 head of swine at the time of the survey.

A total of 3.3 ± 0.1 percent of all breeding females died and 17.5 ± 0.7 percent were culled between December 1999 and May 2000. The most common reasons cited for culling were age, lameness, performance and reproductive failure. Measures of poor performance in this survey included small litter size, high pre-weaning mortality and low birth rate. Other reasons for culling included upgrading herd genetics, poor body condition and liquidation of the breeding

herd for financial reasons. Table B-7 presents the reasons for culling and the relative percentages of swine culled for those reasons.

Table B-7: Reasons for culling swine

Cause	Percent of culled females ± SE	Percent of all females ± SE
Age	41.9 ± 1.8	7.3 ± 0.4
Lameness	16.0 ± 1.2	2.8 ± 0.3
Performance	12.0 ± 0.7	2.1 ± 0.1
Reproductive failure	21.3 ± 1.3	3.7 ± 0.2
Other	8.8 ± 1.6	1.6 ± 0.3

The two most commonly reported health problems in breeding females were roundworms (an intestinal parasite) and Porcine Reproductive and Respiratory Syndrome (PRRS). Swine dysentery was the only health problem more commonly reported on small operations (less than 250 swine) compared to large operations. Other diseases occurred at a higher rate on larger operations. Unfortunately, no data were presented to indicate number or percent of animals affected by disease. Problems at farrowing and other reproductive problems were not reported.

D. Sheep

Most sheep in the US are raised for the production of both wool and meat. In the Eastern US, most sheep are raised on farms in fenced pasture, and may be supplemented with grain. In the Western US, it is more common to maintain sheep on open range. Lambs are generally born in late winter or early spring. Age at slaughter is variable, depending on the price of lamb compared to the price of grain and other inputs.

The National Agricultural Statistics Service (NASS), USDA reported 66,100 sheep operations with a total national herd of 6,965,000 head as of February 2002. The 2001 Reference of Sheep Management in the United States (USDA/NAHMS) reported that 23.8 ± 1.0 percent of rams and 18.3 ± 0.5 percent of ewes in all flocks were culled in 2000, and 5.0 ± 0.1 percent of all sheep and lambs died. Sheep raised on farms had a marginally higher death loss compared to open or fenced range sheep (5.6 vs. 4.5 and 4.7 percent, respectively). Data on culling rates by type of operation were not available. Table B-8 presents primary reasons for culling by sex for animals culled in 2000.

Table B-8: Primary reasons to cull for all rams and ewes culled

Reason for Culling	Rams	Ewes
Age	47.7 ± 2.1[1]	47.9 ± 1.8[2]
Teeth problems	0.8 ± 0.3	5.3 ± 0.5
Poor mothering	N/A	3.3 ± 0.3
Mastitis	N/A	3.3 ± 0.2
Failure to lamb	N/A	5.5 ± 0.4
Ram breeding soundness	13.8 ± 1.4	N/A
Other reproductive	3.6 ± 1.1	1.2 ± 0.4

[1] Percent of all culled rams ± SE
[2] Percent of all culled ewes ± SE

Predators (23.5 ± 1.0 percent), dystocia (12.3 ± 0.5 percent) and old age (15.4 ± 0.8 percent) accounted for 51.2 percent of all adult sheep that died or were lost in 2000. Other problems included respiratory disease, other diseases, digestive and metabolic problems (including milk fever and pregnancy toxemia), poisoning/toxicity, weather, and theft. Table B-9 presents data on major causes of death for adult sheep and lambs that died in 2000.

Table B-9. Causes of death for adult sheep that died in 2000

Cause	Sheep	Lambs
Predators	23.5 ± 1.0[1]	44.1 ± 1.1[2]
Digestive	6.7 ± 0.6	9.9 ± 0.6
Respiratory	7.0 ± 0.8	11.7 ± 0.7
Metabolic	3.7 ± 0.4	1.0 ± 0.1
Dystocia	12.3 ± 0.5	NR[3]
Other disease	3.0 ± 0.2	2.0 ± 0.3

[1] Based on percent of all sheep that died ± SE
[2] Based on percent of all lambs that died ± SE
[3] Not reported

As for swine, incidence and causes of morbidity in sheep was presented as percentage of operations reporting the problem. Data on number or percent of animals affected by illness were not presented in the USDA/NAHMS report. The most commonly reported health problems were stomach or intestinal parasites, clostridial infection, contagious ecthyma (sore mouth), and foot rot. Respiratory and reproductive problems were not reported as causes of illness in sheep or lambs in this report.

E. Goats

Statistics on goat production in the US were not available through USDA/NAHMS. According to the Agriculture Databases for Decision Support (ADDS), there are approximately 2 to 4 million goats raised in the US (http://www.adds.org/CGI-BIN/om_isapi.dll?clientID=23885&infobase=National%20Goat%20Database&softpage=Browse_Frame_Pg). However, no reliable or comprehensive statistics on goat numbers or their production in the US could be found. Goats are generally divided into three distinct types for meat, dairy or fiber (mohair or cashmere) production. Goats grown for meat or fiber are raised predominantly in large herds on open range, while dairy goats are raised in smaller herds on limited acreage with grain feeding. Intestinal parasites and respiratory diseases appear to be the most common illnesses reported in goats, although actual data were not available (ADDS Goat Handbook 1993).

Appendix C:

Comparison of Outcomes Among Assisted Reproductive Technologies (ARTs)

Appendix C: Comparisons of Outcomes Among Assisted Reproductive Technologies (ARTs)

Although there have been several studies comparing the outcomes of somatic cell nuclear transfer (SCNT) with various other assisted reproductive technologies, it is important to note that most of these evaluated data once other technologies had matured and were well-integrated into agricultural practice. The following summary provides an overview of several studies comparing the outcomes of four key ARTs. Comparison of success rates from SCNT with these ARTs may not be entirely appropriate due to the relative newness of SCNT technology. However, a review of the available studies indicates a trend of increasing adverse outcomes with increasing technological assistance; specifically, the increased rate of pregnancy failure, late gestational complications and problems associated with Large Offspring Syndrome (LOS) are most commonly associated with *in vitro* manipulation of the embryo. Table C-1 presents outcomes noted in various studies of artificial insemination (AI), *in vivo* produced embryo transfer (ET), *in vitro* produced embryos (IVP), blastomere nuclear transfer (BNT), and SCNT.

Table C-1. Outcomes noted among studies for various ART in cattle, swine and sheep.

Developmental Node[1]	Gestational Period	ART	Outcome	Reference	Comments
Node 1	Early conceptus, early embryo prior to completion of organogenesis (gd 42 in cattle)	IVP, BNT	Higher rate of embryonic death than AI or *in vivo* produced embryos	Reichenbach et al. 1992; Kruip and den Daas 1997; Wells et al. 1998; Hasler 2000;	Cattle and sheep
		IVP	Pregnancy loss following transfer of IVP or *in vivo* produced embryos prior to gd[2] 21 or within 2 weeks of transfer	Farin and Farin 1995 McMillan et al. 1998	
		IVP	Increased total length of conceptus from IVP embryos 2X that of *in vivo* produced at gd 12 and 17	Farin et al. 2001 Lazzari et al. 2002	

Developmental Node[1]	Gestational Period	ART	Outcome	Reference	Comments
		IVP	Gd 16 IVP conceptuses shorter than *in vivo*	Bertolini et al 2002	Likely reflects survival status during critical time of maternal recognition
		IVP	19% of conceptuses from IVP blastocysts degenerated by gd 17	Farin et al. 2001	
		IVP	Altered embryonic disc morphology affected by culture medium for IVP embryos	Fischer-Brown et al. 2005	
		IVP	Pregnancy rates 45% or higher in dams receiving IVP embryos	Hasler 2000; van Wagtendonk-de Leeuw et al. 2000; Lane et al 2003	Factors affecting outcome include embryo culture system, embryo quality, embryo evaluator, number of embryos implanted, synchrony with dam's estrus cycle, fresh vs. frozen embryos
		ET	Higher embryo survival rate when embryos are transferred fresh rather than frozen-thawed	Spell et al. 2001	
		AI	Embryo loss ~30% by gd 30in beef and dairy cattle	Smith et al. 1982; Sreenan and Diskin 1983; Dunne et al. 2000; Santos et al. 2004	
		AI	Embryo loss by gd 21 associated with high plasma estrogen levels on day of insemination	Shore et al. 1998	Possible estrogenic effect of legume in diet
Node 1	Late embryonic/ early fetal period (days 30-90)	ET, IVP	Embryo loss for *in vivo* produced embryos < 5% (from 2 months – term)	King et al. 1985 Hasler et al. 1987	
		IVP	Embryo loss for IVP embryos higher - 13% after gd 40 - 10.7-13.1% - 24% total between gd 53-calving, with more between gd 50-80	Hasler et al. 1995; Agca et al. 1998; Hasler 2000; Block et al. 2003	Depending on medium

Developmental Node[1]	Gestational Period	ART	Outcome	Reference	Comments
		AI, ET, IVP	Pregnancy rates at gd 22 not different among groups. At gd 42, pregnancy rates similar between AI and ET, but increased embryo loss in IVP compared to AI and ET	Drost et al. 1999	
		IVP	Abnormal development of allantoic membranes and cavity in placentae of IVP embryos gd 30-90	Peterson et al. 2000	Abnormal placental development and reduced placental blood membrane development
		IVP	Abnormal placentome and blood vessel morphology between gd 70-222	Miles et al. 2004 Miles et al. 2005	
		IVP	Gd 61 and older fetuses heavier than ET fetuses; altered fetal organ growth; excessive amniotic fluid	Sinclair et al. 1999	
		IVP	Gd 70 altered angiogenesis and placental morphometry; modified synthetic oviductal medium (mSOF) compared with medium with serum had fewer placentomes, low placental fluid volume and lower fetal weight: placental weight ratio; Placentomes (cotyledon tissue) had decreased density of blood vessels, decreased expression of angiogenic factor mRNA and vascular endothelial growth factor (VEGF)	Miles et al. 2005 Farin et al. 2006	
		AI	Fetal loss by gd 44 30-40% in swine pregnancies	Vonnahme et al. 2002	Fetal survival related to placental efficiency

Developmental Node[1]	Gestational Period	ART	Outcome	Reference	Comments
		AI	Embryonic/fetal loss varies from 10 to 20% between gd 28 and 80 in beef and dairy cattle	Pope and Hodgson-Jones 1975; Kummerfeld et al. 1978; Bulman and Lamming 1979; Lucy 2001	Progesterone levels in dams' milk may be normal through first 30 days of pregnancy, followed by sudden drop
		AI	Embryonic/fetal loss 11 to 44% by gd 50 in beef cattle	Bulman 1979	Attributed to bull
Node 1	Late gestation	ET, IVP	Compensation in vascular beds of IVP bovine embryos; Compared with *in vivo* embryos, IVP had decreased fetal villi, binucleate cell volume densities in placentomes. Proportional volume of blood vessels in maternal caruncles increased in IVP group. Ratio of blood vessel volume density: placentome surface area increased.	Miles et al. 2004	Theorized to compensate for increased fetal size and need for increased nutrients and gas exchanges, but increased vascular blood network at level of placentome
		IVP	IVP fetuses show increased glucose and fructose in fetal plasma levels; increased placental surface area	Bertolini et al. 2004	
		IVP	Hydroallantois frequency in IVP pregnancies (1/200) higher than in "normal" pregnancies (1/7,500)	Hasler et al. 1995	
		IVP, SCNT	Pregnancy loss higher in SCNT than IVP embryos; 50-100% loss gd 30-60; placentae hypoplastic and reduced cotyledonary development	Hill et al. 2000; Chavette-Palmer et al. 2002; Heyman et al. 2002; Edwards et al. 2003; Lee RS et al. 2004	

Developmental Node[1]	Gestational Period	ART	Outcome	Reference	Comments
		BNT	Late gestation abortions, stillbirths, underdeveloped fetuses for gestational age, edema, hydronephrosis, testicular hypoplasia, skull and heart malformations; lack of udder development in dams	Wells et al. 1998	
		IVP, SCNT	Broad distribution of fetal and neonatal body weights for both IVP and SCNT-derived embryos; shifted to "heavy" relative to in vivo embryos	Wilson et al. 1995 Kruip and de Daas 1997 Farin et al. 2001 Miles et al. 2005	Two competing explanations: (1) "normal" for these animals may be heavier than for *in vivo* produced embryos, or (2) a proportion of animals shifts weight distribution of population
			Adaptation to small changes in biochemical parameters and morphology	Sangild et al. 2000	
		IVP, ET	Increased gestation length, dystocia, perinatal mortality, fetal edema, altered organ development, abnormal limbs	Kruip and den Daas 1997; Behboodi et al. 1995; van Wagtendonk-de Leeuw et al. 1998; Farin et al 2001; Bertolini and Anderson 2002; Edwards et al. 2003; Rerat et al. 2005	Frequency and severity of abnormalities: IVP>ET>AI
		AI	55.9% of abortions due to infection	Santos et al. 2004	
Node 2	Perinatal	IVP, BNT	Increased birth weight, Increased crown-rump length; increased mortality and physical deformities	Behboodi et al. 1995; Wilson et al. 1995; Walker et al. 1996; Rerat et al. 2005	
		IVP	Perinatal mortality in IVP ranges from 2.4-17.9%, due to dystocia associated with large fetuses	Hasler et al 1995; van Wagtendonk-de Leeuw et al. 1998; Block et al. 2003	Lower in heifers than in cows

Developmental Node[1]	Gestational Period	ART	Outcome	Reference	Comments
		IVP, SCNT	IVP and SCNT fetuses have cerebellar hypoplasia, respiratory distress, and heart enlargement	Schmidt et al. 1996; van Wagtendonk-de Leeuw et al. 2000; Chavette-Palmer et al. 2002	
		IVP	Altered expression of mRNA for non-imprinted myostatin and glyceraldehydes-3-phosphate in IVP fetuses	Crosier et al. 2002	
		IVP, SCNT	Altered expression of mRNA or protein in IVP and SCNT placentae for VEGF, peroxisome proliferators activated receptor γ, leptin, bovine placental lactogen, transforming growth factor (TGF) β1, 2, 3, TGF- β receptor, major histocompatibility class I antigens	Davies et al. 2004; Miles et al. 2004; Ravelich et al 2004; Miles et al 2005; Ravelich et al. 2005	
		IVP, SCNT	Expression of demethylating enzymes DMT 1, 3a altered in IVP and SCNT preimplantation embryos	Wrenzycki et al. 2004	
		BNT	Birth weight range 26.4 to 67.3 kg; slow to stand, poor suckling behavior, flexor tendon deformities, hypoxemia, hypoglycemia, acidosis, hypothermia; altered metabolic hormones (thyroxine, triiodothironine, and insulin)	Garry et al. 1996	
		BNT	Calving rate ~50% using high quality embryos; some very large calves (up to 70.5 kg); contracture of limbs and spine, cardiac and skull deformities noted in a few calves; high rate of dystocia (52/100); hydroallantois observed in four cows	Willadsen et al. 1991	

Developmental Node[1]	Gestational Period	ART	Outcome	Reference	Comments
		IVP, SCNT	Increased incidence of dystocia and C-section deliveries for IVP pregnancies compared to AI/NM; lack of contractility and other signs of labor in ewes; higher mortality among IVP and SCNT compared to AI/NM	Ptak et al. 2002	Sheep
		AI	Heat stress reduces birth weight and passive transfer of immunity and results in low IgG concentration in calves	Collier et al. 1982	High levels of glucocorticoids accelerates "gut closure"
Nodes 2-3	Postnatal	IVP	Increased feed intake and growth rate	Rerat et al. 2005	
		IVP	Altered glucose and electrolyte metabolism compared to AI persisting through early juvenile period	Rerat et al. 2005	

[1] For the purposes of this table, Developmental Node 1 is divided into three stages of pregnancy: early embryo, late embryo-early fetal, and late gestation.
[2] Gd= gestation day or day of pregnancy.

A. Successes and Failures of AI, IVP, and ET

Success of AI depends on a variety of factors, including health of the female and timing of insemination relative to ovulation. In dairy cattle, conception rates to AI following spontaneous estrus have declined from approximately 55 percent in the 1950s to 45 percent in the late 1990s. The use of hormones to synchronize estrus for timed AI has further reduced conception rates to approximately 35 percent. The reasons for this apparent reduction in dairy cow fertility are not clear, although a number of factors have been cited as possibly contributing to the phenomenon, including increased milk production (resulting in increased stress and reduced availability of nutrients for reproductive function), increased average herd size (resulting in fewer person-hours spent observing cows for estrus behavior), nutrition, herd health, inbreeding, and environmental pollution (Lucy 2001). Embryo loss has been estimated to occur at a rate of 10 to 20 percent in dairy cattle (Lucy 2001) and as high as 30 percent in beef cattle (Dunne et al. 2000), and generally occurs prior to 30 days gestation. Fetal losses in swine pregnancies can be as high as 40 percent following AI (Vonnahme et al. 2002). The reasons for these losses *in utero* are not

always apparent. Lucy (2001) noted that embryo loss may occur even in cases where the developing embryo appeared normal. However, in swine, fetal loss appears to be related to the size and efficiency of the placenta (Vonnahme et al. 2002).

Betts and King (2001) noted that the developmental competence (an embryo's ability to progress through normal cell division and development) of IVP and cultured embryos was low. Using *in vitro* procedures (as published up to 2001), less than half of inseminated bovine oöcytes reached blastocyst stage, and of those that did, many did not implant or attach following transfer. Betts and King (2001) noted that chromosomal abnormalities such as aneuploidy and polyploidy played a fundamental role in most of these embryonic deaths.

The evolution of IVP technology in cattle can be observed by comparing early studies (conducted prior to 2002) with more recent publications. Studies using IVP embryos during the mid- to late 1990s (Behboodi et al. 1995; Farin and Farin 1995; Hasler et al. 1995; Walker et al. 1996; Drost et al. 1999; Sinclair et al. 1999) noted relatively high rates of embryo loss and LOS among fetuses and neonatal calves. In contrast, several more recent studies using IVP embryos have indicated few or no problems (Chavatte-Palmer et al. 2002; Heyman et al. 2002; Bertolini et al. 2004; Rerat et al. 2004). However, high embryonic mortality and placental abnormalities may still be observed with IVP in some labs (Miles et al. 2004; Miles et al. 2005).

Embryo transfer, in which oöcytes are fertilized *in utero* then removed and transferred to surrogates, has become a commercially viable technology (See Chapter II), and is generally more successful than IVP. In a study by Drost et al. (1999), initial pregnancy rates, as determined by blood progesterone levels at gestation day 22, were similar among cows bred by AI, ET or IVP. However, by gestation day 42, embryo loss among cows receiving IVP embryos was higher than either AI or ET, while pregnancy rate was similar between cows bred by AI compared to those receiving ET embryos. The success of ET may be affected by treatment of the embryo prior to transfer and synchrony between the surrogate and the embryo donor (Pope 1988; Spell et al. 2001). According to Spell et al. (2001) fresh embryos had a higher rate of survival than embryos that had been frozen then thawed prior to transfer. Embryo survival was also higher when surrogates had been in estrus within 12 hours of the embryo donor (Spell et al. 2001).

In order to follow fates of client-owned pregnant cows carrying IVP-derived pregnancies in a commercial ET operation, Hasler et al. 1995 noted that for the first 100 transfers, 24 ended in pregnancy loss before 100 days of gestation. The success rate improved the subsequent year, however, with only 7 percent of IVP-derived pregnancies spontaneously aborting. They compared these results to 5.3 percent of ET pregnancies aborted between two and seven months of gestation in an earlier study.

In a comparison of AI and IVP, Behboodi and coworkers (1995) noted an increased incidence of dystocia and Cesarean sections (C-section) for IVP derived pregnancies compared to AI in a small group of cattle (8/13 IVP-derived pregnancies vs. 7/71 AI pregnancies requiring C-section). Birth weights of calves derived from IVP embryos were higher than calves produced by AI, likely contributing to the observed increase in dystocia among dams carrying IVP-derived pregnancies. Sinclair et al. (1999) also observed large IVP-derived fetuses with altered development and excessive amounts of amniotic fluid. In that study, nine of 13 fetuses (69 percent) derived from embryos co-cultured with granulosa cells (a type of cell found in the ovary) and one of six embryos (17 percent) incubated in synthetic oviductal fluid (SOF) plus steer serum were oversized, while embryos that had been incubated with SOF alone produced normal sized fetuses. Bovine embryos cultured for three to five days post-fertilization also were associated with increased dystocia due to oversized calves in a study by Walker et al. (1996). (See discussion of influence of culture conditions on success rates in Chapter IV).

Farin and Farin (1995) collected bovine IVP and ET fetuses from beef heifers at seven months gestation and compared development between the two groups. Fetuses from the IVP group were heavier than their ET counterparts (18.6 ±1.1 vs. 15.4 ±0.8 kg), had greater heart girths (56.5 ± 1.2 vs. 52.4 ± 0.9 cm) and weights (139.7 ± 8.3 vs. 116.2 ± 5.8 g), and greater long bone lengths (23.1 ± 0.6 vs. 21.3 ± 0.4 cm).When organ and skeletal measures were compared on a per kilogram body weight basis, however, IVP fetuses had consistently smaller skeletal measures than ET fetuses. Internal organ weights per unit of body weight were not different between the two groups of calves. The authors concluded that IVP fetuses were undergoing abnormal and disproportionate development compared to ET fetuses. It should be noted that the most rapid period of prenatal growth in cattle is during the last two months of gestation (months 8 and 9) (NRC 2001), which would have occurred after these pregnancies were terminated.

Young and Fairburn (2000) noted that both IVP and embryo culture have resulted in abnormal phenotypes, including up to two-fold increases in birth weight (LOS), excess amniotic fluid, hydrops fetalis[116], altered allometric organ growth[117], advanced fetal development, placental and skeletal defects, immunological defects, and increased perinatal death.

Markette et al. (reviewed by Farin et al. 2001) observed that 54.7 percent of ET recipients were pregnant at 60 days gestation, with the majority of pregnancies lost prior to day 24 of gestation.

[116] Accumulation of fluid in the entire body of the newborn.

[117] Allometric growth refers to differences in the rate of growth of a particular organ or part in relation to the rest of the organism. An example of normal allometric growth is the legs of a newborn foal (horse) in proportion to its body size; the legs are long and out of proportion to the rest of the body. As the foal ages, the body grows (and fills out) more rapidly than the legs, so that in adult horses the legs appear proportional to the rest of the body. Altered allometric growth in the context of ARTs has resulted in enlarged hearts and undersized kidneys, as well as other organs, which are not appropriately proportioned to the rest of the body.

In a large study, King et al. (1985) reported that the incidence of pregnancy loss in 1,776 embryo transfer recipients was 3.15 percent from 2 to 3 months of gestation, and 2.14 percent between 3 to 7 months. These mid- and late-gestation spontaneous abortions were not influenced by embryo age, embryo quality, time between embryo collection and transfer, asynchrony of recipient with donor estrus, donor age, ovarian response to gonadotropin treatment, or whether or not the donor had a history of infertility, according to the authors. In most studies, pregnancy loss during the fetal period (day 42 to 280 of gestation in cattle) was greater following transfer of embryos produced *in vitro* than that for embryos produced *in vivo*. Mid- to late-gestation spontaneous abortion of about 7 to 13 percent has been reported for recipient cattle carrying fetuses derived from IVP embryos, and in some studies pregnancy loss has been considerably higher (Farin et al. 2001).

Conversely, Bertolini et al. (2004) compared fetal development in *in vivo* and IVP cattle pregnancies and reported no significant difference between groups for pregnancy rates (20/53 and 36/112 for control and IVP groups respectively) and fetal losses after day 45 (2/20 and 3/36 for control and IVP groups respectively). They did report that fetal losses between gestation days 30 and 44 were 3.4-fold higher ($P<0.05$) in the IVP group (17/36) than in controls (4/20). Also in contrast to earlier studies, Bertolini et al. (2004) reported that their measurements of conceptus physical traits for both *in vivo* produced controls and IVP pregnancies on days 90 and 180 demonstrated allometric proportionality between fetal body size and body weight with no physical deformities observed in any fetus.

In a review of research on early embryo development, Gardner and Lane (2005) stated that the environment of the preimplantation embryo has a profound effect on the physiology and viability of the conceptus. Among the many factors that can influence development of IVP embryos, they cite the use of serum products as an important contributor to developmental abnormalities in cultured embryos. These authors state: *"Mammalian embryos are never exposed to serum* in vivo*...Rather, serum is a pathological fluid, the composition of which is greatly undefined and varies enormously with source…serum induces premature blastulation in domestic animal embryos…affects embryo morphology…and leads to perturbations in ultrastructure…and energy metabolism."* Other factors that may influence development of embryos *in vitro* include ammonia, oxygen, inadequate nutrients, and freezing (Gardner and Lane 2005).

Rerat et al. (2004) compared the perinatal health characteristics of IVP and AI cattle and observed no differences in post-natal mortality or viability. Calves in this study were generally healthy with the health status of IVP calves at birth and during the first 112 days of life similar to that of AI calves. Clinical traits such as heart rate, rectal temperature, and respiratory rate were nearly identical in both groups. At birth, measurements indicative of growth performance such as potassium, 3,5,3'-triiodothyronine (a metabolic hormone), and thyroxine concentrations were

lower in IVP than in AI calves. Postnatally, IVP calves had a faster growth rate than AI calves under conditions of identical nutrient intake.

Sakaguchi et al. (2002) induced twinning in Japanese beef cows by transferring one or two *in vivo* fertilized embryos into AI bred cows. Fetal dystocia occurred in 7 of 14 twin parturitions, in which some twin calves appeared to enter the uterine cervix at the same time, but no single parturition was accompanied by dystocia. The incidence of retained placenta was significantly higher in the twin parturitions (10/14; 71 percent) than in the single parturitions (2/22; 9 percent). These complications are known to occur with natural twins in cattle, however, and may not be directly related to ET technology. The incidence of retained placenta in healthy, single calf-bearing dairy cows is approximately 5-15 percent, (slightly lower in beef cows) and is increased when there are twins. The expected incidence of dystocia is 10-15 percent in first-parity animals, and 3-5 percent in mature cows (Merck Veterinary Manual Online 2002).

B. Outcomes for BNT, Fetal- and Adult-Cell SCNT

Although success rates for various types of cloning have improved, they are still highly variable across studies. In earlier studies, generally less than 10 percent of all NT embryos transferred to recipients were born alive (Wells et al. 1999). Some of these early studies noted that both blastomere and somatic cell NT clones appeared to have the same low success rate and exhibited many of the same problems, such as poor or dysfunctional placentation and LOS (Stice et al. 1996; Wells et al. 1998). Stice et al. (1996) reported that no fetuses derived from BNT survived beyond day 60 of gestation. Wells et al. (1998) reported a 64 to 80 percent pregnancy loss during the attachment phase for clone fetuses derived from an embryonic sheep cell line, while a further 43 percent of pregnancies were lost in the last trimester, such that 11 percent of embryos survived to term (12/112). In contrast, Le Bourhis et al. (1998) reported 9/30 transferred male bovine BNT clones developed to calving, while 6/27 female BNT clones resulted in live calves. Heyman et al. (2002) compared development and survival of BNT, fetal and adult NT clones to IVP-derived embryos under the same culture conditions. Pregnancy loss from 90 days of gestation to calving were 43.7 percent for adult and 33.3 percent for fetal SCNT, compared to 4.3 percent for BNT clones, while none of the IVP-derived pregnancies were lost. Pace et al. (2002) reported 75 percent pregnancy loss of adult (some transgenic) SCNT embryos throughout pregnancy.

Results from these studies may reflect the evolution of NT technology over time. Embryonic or BNT cloning and IVP success rates appear to have improved. Although losses remain high for the newer SCNT technology, success rates for this technology also have improved over time. It remains to be seen what progress may be made in further reducing pregnancy loss and other risks associated with SCNT.

C. Conclusions regarding outcomes for ARTs

Based on the studies reviewed, there appears to be a general trend indicating that the frequency of embryo/fetal loss and abnormal pregnancy outcomes increases with increasing manipulation of the embryo and *in vitro* culture. This trend is evident, even when maturity of the technology is considered. Causes of embryo/fetal loss are not always evident, but late gestational complications (hydrops and dystocia) and fetal/neonatal abnormalities (skeletal and organ deformities, oversize, metabolic alterations) have all been noted in ET, IVP, BNT and SCNT. The frequency of these outcomes varies somewhat among laboratories, but has the general trend ET<IVP<BNT<SCNT. These data support a conclusion that SCNT falls on a continuum of ARTs, and that the adverse outcomes noted with SCNT are not unique, but are of concern due to their increased frequency.

Appendix D:

Transgenic Clones

Appendix D
Transgenic Clones

A. Issues

The current risk assessment is limited to address the risk of clones from non-transgenic cells. Although not within the purview of this analysis, the results of a number of studies that address either transgenic animal clones or transgenic and nontransgenic animal clones (Hill et al. 1999; the "ACT series" including Cibelli et al.1998, Lanza et al. 2000, and Lanza et al. 2001 for cattle; McCreath et al. 2000 and Denning et al. 2001 in sheep; Baguisi et al. 1999 and Keefer et al. 2001a in goats; Carter et al. 2002, Lai et al. 2002, and Lee GS et al. 2003 in swine) are presented here to clarify the relative utility of such studies for assessing potential risk(s) associated with non-transgenic somatic cell nuclear transfer (SCNT).

Many reviews of SCNT outcomes cite these papers as indicative of the severity of adverse outcomes associated with cloning, or a demonstration of the positive outcomes that can come from cloning. Hill et al. (1999), in particular, is often cited as the seminal "adverse outcome" paper for cloning. On the other hand, if only the "final report" paper of the ACT series, Lanza et al. 2001, were read, it would not be possible to know that the cells from which the cattle were cloned were indeed transgenic. This distinction only becomes apparent when the earlier papers are also reviewed. Most recently, headlines were generated when Pearson (2003) reported on "sudden death syndrome" in pig clones; CVM reviewed the paper that describes their generation and noted that these pig clones carried two distinct transgenes (Lee GS et al. 2003).

Because these animals are transgenic clones, it is not possible to determine whether adverse outcomes result from the direct effect of the expression of the transgenic construct, pleiotropic effects resulting from insertion of the construct, the SCNT process, or some interaction of any or all of these processes. For example in a comparison of 34 cells lines, Forsberg et al. (2002) reported that when otherwise similar cells are used as donors for SCNT, those that are transgenic (for a selectable marker gene) result in lower pregnancy initiation (22 percent vs. 32 percent) and calving (3.4 percent vs. 8.9 percent) rates. The authors hypothesize that the additional culturing required togenerate transgenic cells, selection of transgenic lines, or the DNA construct itself, could be responsible for the lower rates.

CVM thus assumes that transgenic clones occupy a different "risk space" from "just clones." Conversely, an argument can be made that if no adverse outcomes are detected, then for these animals, neither process sufficiently perturbed development to induce anomalies. We have

included those studies in the overall risk assessments if such results were obtained. Nonetheless, because these studies occupy such a large segment of the cited literature, a few are presented here to illustrate the range of responses noted, with the appropriate caveats for interpretation.

B. Cattle

Hill et al. 1999

Hill et al. 1999 reports on a group of 13 transgenic clones of a Holstein bull. Twelve Brangus cows carrying 13 fetuses cloned from Holstein cells were originally included in the study, although three of these transgenic clone fetuses died prior to the perinatal period (defined in this paper as two weeks prior to anticipated delivery and a few days thereafter), and one cow aborted at eight months of gestation. Two cows developed hydroallantois and were delivered by C-section; four others were also delivered by this method due to subjective judgment regarding fetal size. The two remaining pregnancies delivered vaginally. Birth weights of the transgenic clones ranged from 44-58.6 kg (average Holstein male calf weight is in the range of 40-50 kg), and cited as within the weight range of *in vitro* produced embryos. Five of the eight live born clones were judged to be normal within four hours of birth based on clinical signs and blood gas measurements. Three of the eight were immediately diagnosed with neonatal respiratory distress. One of these calves died from pulmonary hypertension, pulmonary surfactant deficiency, and elevated systemic venous pressure at day 4. The other three animals recovered. Two of the five fetuses that did not survive to birth also exhibited signs of pulmonary hypertension and placental edema at necropsy. Another clone died at 6 weeks of age with signs of respiratory distress; subsequent field necropsy suggested dilated cardiomyopathy, although no definitive diagnosis could be made.

The Advanced Cell Technology Series

The series of papers from the Advanced Cell Technology group (Cibelli et al. 1998, Lanza et al. 2000, and Lanza et al. 2001) on the health of clones are similar to that of Hill et al. (1999) in that the animals presented are clones that are derived from transgenic cells. Interpretation of any adverse outcomes is thus also confounded by the potential role of the transgene and its insertion.

The results of these studies are summarized in Lanza et al. (2001), in a short overview with accompanying supporting documentation provided by the journal in electronic form. Of 30 fetal transgenic clones that developed to term, 24 were reported healthy at 1-4 years of age, but five died within 7 days of cardiopulmonary difficulties that the authors speculated were secondary to placental insufficiencies. The sixth animal died at day 149 due to enteric disease, lymphadenopathy, and exhibited mild placental edema and high fever at birth. Problems observed at birth included placental edema, including edematous cotyledons (attachment sites of the placenta to the uterus), labored breathing, froth and fluid in the lungs, pulmonary edema,

pneumonia, high fever, septicemia, lethargy, abdominal distention, masses in the abdomen, liver damage due to hypoxia, and heart abnormalities.

Birth weights of the survivors were reported as 45 ± 2 kg (this paper cites normal as 43 kg). An unspecified number exhibited pulmonary hypertension and respiratory distress at birth. Presumably, they received supportive care at that time. Another unspecified number were also reported as experiencing fever following vaccination. This is not an atypical response among calves receiving vaccinations, as stimulating a potent immune response is likely to produce at least a mild local and systemic (fever) reaction in the animals (Roth 1999).

Physical and veterinary examination of surviving animals aged 1-3 years were reported as normal and included temperature, pulse, and respiratory rate. No abnormalities were detected in general appearance, on auscultation (listening to breathing, heart beat, and digestive sounds), and behavior appeared normal. Puberty onset was reported to occur at the expected time, and fertility appeared to be normal. At the time of publication (2001), two of the animals had delivered apparently normal progeny.

Clinical chemistry parameters evaluated for these animals included electrolytes, urea, creatinine, glucose, bilirubin, aspartate aminotransferase (AAT), sorbitol dehydrogenase (SGT), albumin, globulin, and total protein. Globulin and total protein measurements were reported in the publication as "slightly below normal." All other measurements were reported to be within normal range. Hemograms (analysis of cellular components of blood) were all reported as normal: hematocrit, hemoglobin, red blood cells, mean red cell volume, mean red cell hemoglobin concentration, and white blood cell numbers and differentials were within normal ranges. Blood gases were also within normal ranges. To examine immunocompetence in the clones, peripheral blood lymphocytes from the transgenic clones and conventional Holsteins were compared to determine whether the same ratio of cell surface markers were present, and if the transgenic clone cells responded to mitogen challenge in the same way as cells from conventional Holsteins. No significant differences were observed between the cell surface markers or cellular responses of cells from conventional animals or clones.

In the early spring of 2003, an interview of an ACT executive reported in the lay press indicated that two animals from this cohort had developed significant health problems. One animal was reported to have developed a tumor, and the other was diagnosed as having neurological problems. The first animal apparently died during surgery to remove the tumor, and no further information is available on the potential causes of the tumor. The second animal was later diagnosed as being positive for Johne's disease (*Mycobacterium paratuberculosis)*, an infectious, chronic, progressive disease that often presents with chronic diarrhea and eventual cachexia (general physical wasting and malnutrition). It is therefore unlikely that this animal's symptoms

were due to either cloning or transgenesis. We are unaware of any other adverse outcomes associated with these animals.

C. Swine

Carter et al. 2002

Carter et al. 2002 reported on the overall health status of transgenic swine clones produced from cells transfected with green fluorescent protein (GFP). The 10 transgenic piglet clones from three litters were followed for the first six months of life.

Five of the ten transgenic swine clones died or were euthanized during the study. Two piglets died of congestive heart failure at 7 and 35 days of age, two others died from bacterial infections at 3 and 116 days of age. The fifth animal died at 130 days of age, following a history of chronic diarrhea, decreased growth and vitamin E deficiency. The remaining five piglets were reported as healthy and growing similarly to conventional animals housed in the same facility at the conclusion of the study. Behavior was reported as "consistent with pigs of their age group."

Average birth weight of the transgenic clones (1,312 g) was similar to average birth weights of conventional piglets from similar genetic background (1,450 g). Average daily weight gain for transgenic clones through the first 16 weeks was (461 g) relative to the herd average (594 g), which the authors considered as within the normal range.

Some of the piglets displayed physical defects. These included two piglets with contracture of the flexor tendons, another piglet with five digits on a forelimb (four digits are normal) and an enlarged dewclaw. Another piglet with low birth weight was described as having short legs and a large, round chest.

Hematology and blood clinical chemistry data were collected beginning at 2 days of age and every two to four weeks until 24 weeks of age. Most hematological variables were similar to the comparator group, except for hemoglobin, hematocrit, and plasma total protein. Mild anemia and low blood protein concentration were observed for the first four weeks, but both these conditions resolved by eight weeks of age. The authors stated that decreased hematocrit and hemoglobin values are common in piglets reared in confinement, and that these symptoms are generally treated with iron dextran. Similarly, clinical chemistry results indicated decreased levels of albumin and globulin during the first four weeks in the transgenic clones relative to comparators, but these values were back within the normal range by eight weeks of age. The authors attributed the decreased protein and globulin values to the decreased colostrum intake of the newborns as the surrogate sow bearing them did not initiate normal lactation, and piglets were dosed with colostrum at some unspecified point after birth.

Seven of the transgenic clones were evaluated for cardiac function. Although no physical defects were found, one piglet had evidence of mitral insufficiency (a condition in which the mitral valve of the heart does not close all the way during contraction, resulting in regurgitation of some of the blood in the left ventricle), and dilation of the left atrium and ventricle. This piglet and two other clones had reduced cardiac output values compared to control piglets, but did not display clinical signs of cardiac disease. Although similar cardiac abnormalities have been noted in conventional swine, the incidence is reported to be very low (Carter citing Hsu et al. 1982). These developmental defects appear to be similar to those noted in cattle clones (see Critical Biological Systems discussions).

Lai et al. 2002

This study was reported in a brief communication, and a limited amount of data was presented. Piglets were generated from cell lines (derived from inbred miniature pigs) in which the α-1-3-galactosyltransferase gene was interrupted by the insertion of a gene sequence in order to create α-1-3-galactosyltransferase "knock-outs." The α-1-3-galactosyltransferase gene codes for a protein that causes hyperacute rejection of swine organs when transplanted into primates. "Knocking out" the expression of this gene increases the suitability of these animals to be used as donors of organs for human transplant patients.

Six piglets were born from two litters. All but one of the piglets had low birth weights compared to the breed average (115 to 650 g vs. 860 g). One piglet from each litter died shortly after birth from what the authors termed "respiratory distress syndrome." A third piglet died at 17 days of age during a routine blood draw, and was diagnosed at necropsy with a dilated right ventricle and thickening of the heart wall. Other abnormalities noted in these surviving transgenic piglets included flexor tendon deformities in three animals; abdominal ascites, enlarged right ventricle, pulmonary hypertension in one animal; and ocular defects and lack of patent ear canals in another animal. The authors attributed these abnormalities to failures in reprogramming during the SCNT process rather than the genetic engineering process, as they did not see a consistent phenotype across the piglets.

Lee GS et al. (2003)

Recently, Pearson (2003) reported that the University of Connecticut laboratory that had generated four transgenic swine clones had announced that the three (of four) surviving piglets died suddenly of heart failure at less than six months of age. The fourth piglet died at three days due to infection and abnormal spine development (Lee GS et al. 2003). Because of the transgenic nature of the animals (they carried genes for human clotting factor IX and porcine lactoferrin, an iron transport protein found in blood), it is not possible to attribute the deaths solely to cloning. It

is unknown whether any cardiac abnormalities were detected in these animals prior to their deaths, or if any measurements of cardiac function were made.

D. Sheep

Denning et al. 2001

Denning et al. 2001 were unsuccessful in producing viable knock-out sheep lacking either the α-(1,3)-galactosyl transferase (GGTA1) or the prion protein (PrP) gene using gene targeted fetal fibroblasts and SCNT. Reconstructed embryos were either incubated for six days (n=48) or overnight (n=93) in synthetic oviductal fluid with bovine serum albumin (concentration not specified). Embryos incubated overnight *in vitro* were then embedded in 1 percent agar chips in phosphate buffered saline and transferred to the ligated oviduct of an estrus-synchronized ewe for six days. A total of 120 morula or blastocyst stage embryos were transferred to 78 estrus-synchronized Finn Dorset ewes as final recipients. It is not clear from this paper how many of the transferred embryos had been incubated *in vitro*. Although 39 pregnancies were diagnosed at gd 35, only eight were maintained to term, resulting in four live births. Three of the four live-born lambs died shortly after birth. The fourth lamb survived 12 days before it was euthanized after developing dyspnea (difficulty breathing) due to pulmonary hypertension and right-sided heart failure. The authors attributed the abnormalities observed to the nuclear transfer procedure, as they were similar to results obtained with non-transgenic NT lambs.

McCreath et al. 2000

McCreath et al. 2000 inserted a promoter-less neomycin selectable marker between the ovine α1(I)-procollagen translational stop and polyadenylation signal[118] in male and female ovine fetal fibroblast cultures. Four transgenic female fibroblast cultures were selected as nuclear sources for SCNT, due to their vigor and normal chromosome number. A total of 80 morula and blastocyst stage embryos were transferred to recipient ewes. No description of post-fusion incubation or estrous cycle status of recipient ewes was provided in this report. Fourteen lambs were born alive; seven of these lambs died within 30 hours of birth. Four more lambs died between 3 days and 12 weeks of age. Three lambs survived and were described as thriving at one year of age. Necropsy of lambs that died *in utero* or after birth revealed a number of abnormalities including a high incidence of kidney defects (frequently renal pelvis dilation) and liver and brain abnormalities (not specified). The authors attribute these abnormalities to either cell treatment or the NT procedure, because the necropsy findings were similar to a previous nuclear transfer study using the same cell lines.

[118] These are regions of the DNA construct that provide instructions for the appropriate processing of information in order to make functional proteins.

E. Goats

Baguisi et al. 1999

In this study from Genzyme Transgenics, six cell lines were established from 35- and 40-day old fetuses that resulted from the mating of a transgenic buck (carrying a human antithrombin III (hAT) gene with a goat β-casein promoter) to a non-transgenic doe. This study differs slightly from several other transgenic cloning studies reported here, in which the gene was inserted into the cell lines before the cultures were established. Clone embryos were cultured on goat oviduct epithelial cells for 48 hours (2-16 cell stage) before being transferred to estrus synchronized recipient does. Although overall cloning efficiency was low (3/112 embryos transferred resulted in live births), all pregnancy losses occurred prior to 60 days of pregnancy. There were no stillbirths and no abnormalities observed in the live-born kids. Kids weighed between 2.35 and 3.5 kg, within the normal birth weight for dairy goats, and are reported as healthy.

Keefer et al. 2001a

In this study from the Nexia Biotechnologies laboratory, goat fetal fibroblasts were transfected[119] with green fluorescent protein (eGFP) and neomycin resistance genes. These are commonly used as markers to demonstrate that transgenes have been inserted and are being expressed. Twenty seven NT embryos were produced with the transfected cells, and an additional 70 non-transgenic NT embryos were constructed and transferred into 13 estrus synchronized recipient does. The authors did not specify how many embryos (transgenic or non-transgenic) were transferred to each doe. Five non-transgenic male clones and one transgenic female clone were born alive. Three of the non-transgenic clones died of bacterial infections, but the single female transgenic clone lived and showed no signs of abnormalities. The kids were all within the normal birth weight range (1.5 to 3.1 kg) for goats at that facility, and no abnormalities were observed in the placentae.

Behboodi et al. 2004

The authors compared development of embryos cultured with oviductal cells *in vitro* vs. embryos cultured *in vivo*. Embryos were constructed using skin fibroblasts of transgenic goats. Only embryos cultured *in vivo* resulted in pregnancies. Two of these pregnancies were lost early in gestation (after 30 days gestation), and four other pregnancies were carried to term. Two surrogate does delivered stillborn kids 2-3 days after their due dates; the other two does delivered healthy kids (one per each doe) at term. The two live clones weighed 3.8 and 4.1 kg at birth, and were within the normal birth weight range for their breed (Saanen). Clones were weaned at 8 weeks of age, and had similar growth rates compared to age-matched AI derived Saanen kids born at the same facility (14.5 and 18.1 kg for clones vs. 14.88 ± 1.98 kg for AI comparators).

[119] Transfection is the process of introducing exogenous DNA into cells without the use of viral vectors. Common methods include co-precipitating DNA with salts and polymers.

Pathology on the dead fetuses indicated diffuse atelectasis (lung collapse) and the presence of amniotic fluid in the lungs. No bacterial or viral cause for the deaths of these clones could be identified. The presence of amniotic fluid in the lungs suggests that the clones attempted to breathe prematurely, a sign of fetal stress which sometimes occurs around the time of birth.

Behboodi et al. 2005

The authors evaluated health, growth, reproduction and lactation in four female goat clones generated from two transgenic fetal cell lines (one cell line coding for glycosylated and the other for non-glycosylated protein). A total of seven clones were carried to term. One clone from the glycosylated group was still born with evidence that the umbilical vessel had ruptured. Two clones died at birth (one from each of the transgenic lines) after failing to breathe on their own, despite attempts at manual ventilation. Thus, two clones from each transgenic line survived to adulthood. There were no differences in birth or weaning weights among the four surviving clones or their age-matched comparators. Transgenic clones exhibited enlarged umbilical stumps (two live and one stillborn kid), "tendon laxity" (three of the four live-born clones), and minor generalized edema (number of clones affected not indicated). These conditions resolved without intervention. The four does were bred and produced nine kids, compared to five kids produced by comparators. Clones expressing the glycosylated version of the protein lactated only briefly, but the does expressing the non-glycosylated protein had normal lactation length and milk yields.

This study is the only one we encountered that presented hematology and blood clinical chemistry data for four goat clones. These data are presented in comparison to four age-matched comparators and values from the literature (Pugh 2002). It is unclear whether or not the comparators in this study were also transgenic, whether they were the same breed as the clones, or how they were generated. Hematology values were similar between clones and comparators, and all hematology values fell within the published range. For clinical chemistry, 18/24 values were not significantly different between clones and their age-matched comparators. Of the 19 clinical chemistry values for which published ranges were available, 18 of the values for clones and comparators fell within the published range. The one value out of the published range was creatine kinase (244.6 vs. 204.4 IU/L for clones and comparators). However, values between clones and comparators were not statistically different. The study does not specify the age of the goats at time of blood sampling, so it is difficult to interpret the high values for CK in these animals compared to the published range.

This study is unique among reports of goat clones because it is the first to indicate possible signs of LOS in goat clones (enlarged umbilici, failure to initiate breathing, tendon problems). It is interesting to note that similar signs have not been noted in non-transgenic goats. We should also note that clinical signs in the four surviving clones resolved, and their health, growth,

reproduction, and hematology, clinical chemistry values indicate that even these transgenic clones are apparently normal.

Landry et al. 2005

The authors reported on growth (weight gain, wither and hip height change) and endocrine profiles of two lines of transgenic goat clones. Group 1 consisted on five does carrying the AT-III gene with a β-casein promoter inserted into cells of a female Toggenburg (dairy breed). The gene inserted into the second line of goats (Group II; n=2) was not identified, but the cells used for cloning were from a female Saanen (also a dairy breed). Non-transgenic, non-clone comparators (n=7) were Boer X Spanish crossbred meat-type does (Group III). The authors did not report on overall health of the clones. One female in each group of clones died prior to the end of the study; one died due to an accident, the other due to a ruptured abomasum. Neither death appears to be related to cloning. Both groups of clones were within range for their breed for birth weight, and appeared to grow normally. Interpretation of hormone profiles (GH, IGF-I, T3, and T4) is difficult due to the fact that the clones and comparators were of different breed (purebred vs. crossbred) and type (dairy vs. meat) backgrounds. However, for most of the hormones assayed, the values for clones fell within the range of values for comparators. The one exception is insulin, which resulted in an extremely low value in blood samples of comparators, and may have been the result of difficulties with the assay.

Melican et al. 2006

With the long-term goal of producing caprine milk containing recombinant therapeutic proteins, the authors demonstrated successful reproduction and lactation in transgenic dairy goats produced by SCNT. Does were produced using primary fetal cells harvested from day 35-40 fetuses. These cells were co-transfected with DNA fragments encoding the heavy and light immunoglobulin chains of three different monoclonal antibodies, plus neomycin resistance as a selectable marker. Two transgenic does were hormonally induced to lactate at two to three months of age and produced small amounts of milk (10-20 ml per day) for 30-40 days. These does were subsequently bred and, at 18 months of age, gave birth to 6 kids (4 were transgenic). Following parturition, the 2 transgenic does produced an average of 2.2 liters of milk per day for 3-6 months. A third transgenic doe, at age 23 months, also lactated following the birth of one transgenic kid, producing 2.6 liters of milk per day for one month prior to being dried off. The milk of all 3 transgenic does contained the antibody for which they were transgenic. These results demonstrate reproductive capacity in cloned female goats, and the ability of these goats to produce normal quantities of milk following parturition.

F. Conclusions Regarding Transgenic Clones

The experience of these cohorts of transgenic clones can be summarized as follows:

- A relatively large fraction of transgenic fetal bovine clones in cohorts surviving to late gestation presents with severe and often fatal difficulties. Some of these are qualitatively similar to those observed in cattle and sheep clones that are not derived from transgenic cells. Due to the many other variables that have been altered in the generation of these animals, at this time it is not possible to attribute these abnormalities to either of the processes (cloning or transgenesis) or their combination.

- Some animals in both cattle cohorts are born with varying degrees of initial respiratory or other physiological distress. Supportive care appears to allow most of these animals to survive to adulthood, although some animals that initially survive can succumb to possible sequellae up to six weeks later.

- Animals surviving to adulthood in the ACT cohort that appear to be healthy on visual inspection also exhibit physiological values that generally fall within normal ranges. CVM is unaware of an update of the health status of the Hill et al. cohort.

- Animals in the ACT cohort surviving to reproductive maturity appear to be capable of bearing normal offspring, although it is not clear whether the offsprings' health has been examined in a rigorous manner.

- Two severe adverse outcomes have been noted for the ACT cohort. Both cloning and transgenesis can likely be ruled out as causes for one (Johne's disease) and no causal agent or process has been associated with the neoplasm found in the other.

- The appearance, behavior, and physiological function of the animals that survive suggest that even the "riskiest" set of clones (*i.e.,* transgenic clones) can develop into normally functioning animals. These results are consistent with the analysis of non-transgenic clones, and provide additional confidence that rigorous monitoring and responsible husbandry of such animals can allow for the selection of animals that are healthy.

- Abnormalities for transgenic sheep clones appear similar to reports for non-transgenic sheep and cattle clones.

- Goats appear to suffer fewer adverse effects compared to sheep and cattle. Of the reports reviewed, only one cohort exhibited clinical signs of LOS.

- Abnormalities reported for transgenic swine clones are similar to those reported for transgenic and non-transgenic cattle clones. CVM is aware of only one report in non-transgenic swine clones (Park MR et al. 2005) in which clones exhibited similar health problems; however, *in vitro* methods used in this study likely influenced the outcome of swine clones in this study.

Appendix E:

The Cyagra Dataset

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Appendix E: The Cyagra Dataset

A. Response to CVM Data Requests

The Center for Veterinary Medicine (CVM) has presented its proposed risk assessment approach at several public venues since the fall of 2002. As part of the proposed approach, we have requested that investigators engaged in cloning cattle, swine, sheep, or goats share data that they might have on the health status of these animals with the Center. The intent of the data request was to supplement the published data with unpublished data generated by the developers of these animals. We thought that data on the health status of animal clones would likely be in the hands of the private sector, which might have less impetus to publish than academic laboratories. The Center promised producers that, to the extent allowed by law, if they wished, their identities could be kept confidential by FDA, and that we would not publish the specific identity and location of the animals.

We discovered that there are very few datasets describing the health of animal clones. In general, clones are monitored closely for the first few weeks of life (or through weaning). They are often then moved from "research/hospital" facilities to "farm-like" facilities, where they are often reared with conventional animals. Most producers kept fairly cursory veterinary records unless the animal was in distress. Further, because of technical issues associated with generating successful pregnancies, only a few clones tend to be delivered at one time or from one cell line. The result is that aggregating and analyzing data becomes difficult unless publications are planned in advance.

As many of the clone producers either have primary employment as academics, or continue to maintain academic appointments, there may be data available that have not yet been shared with CVM because of the investigators' desire not to jeopardize their ability to publish in peer reviewed journals. Because of CVM's pledge to be fully transparent in this risk assessment, we determined that all data submitted would be made public through the risk assessment. We obtained the express permission from the submitters of data for the public release of this data. "Publishing" this information in the assessment could preclude formal publication in a peer reviewed journal, as most high quality peer reviewed journals have a policy of being the site of first publication.

As our risk assessment methodology evolved, it was presented at public fora (the Pew Initiative for Agricultural Biotechnology's September 2002 Biotech in the Barnyard conference, the April 2003 American Registry of Professional Animal Scientists meeting in Maryland, and the FDA

Science Forum of April 2003). Subsequent discussions between clone producers and agency staff resulted in investigators returning to the field to try to collect existing data, or, in one exceptional case, to generate *de novo* data on the health status of clones. Without exception, every clone producer or investigator contacted was willing to answer questions on aspects of clone production, gestation, delivery, and care. Many have provided data or information that we have incorporated into this risk assessment or will use in future iterations. In order to issue the risk assessment in a timely manner, however, we have had to put off our analysis of some of the datasets until the next revision of the risk assessment. We are very grateful to those producers and owners who voluntarily expended significant time, effort, and in some cases, capital, to provide information to us.

B. Cyagra Dataset

One clone producer, Cyagra Inc.,[120] has been engaged in the production of cattle clones since 1999. In the late spring of 2003, Cyagra submitted an extensive database to CVM for use in the animal health component of our food consumption and the animal health risk assessments. These data were made available for CVM to use in our risk assessments with no restriction, except to protect to the extent allowed by law the identity and location of the animals, and their current owners. In order to accommodate this request, CVM issued each animal in the study a unique identification number. These numbers have been employed throughout this analysis.

Cyagra has asserted that they have provided data on all of the clones that they can trace, including those that died, or were euthanized or culled. Animals were divided into three age cohorts by Cyagra: neonates (within 24 hours), 1-6 month age cohort (between 30 and 175 days of age), and 6-18 month age cohort (187-557 days of age).

The age spread among these animal cohorts reflects key stages in physiological development of cattle. For example, digestion differs significantly among different age groups: a 2-month-old calf is just starting to use its rumen, while a 6-month-old calf is a fully developed, cud-chewing ruminant. In this case, these two calves have been grouped together even though they have substantive physiological differences, because they have more in common than, for example, a neonate and a six month old calf. For the sake of accuracy, we have classified this group as 1 to 6 months old. A 6-18 month old calf is not quite old enough to be considered an adult, as it is still growing, and the younger animals in this group will still be pre-pubertal. We have therefore decided to classify this group simply as “6 to 18 months.” The distribution of animals in the cohorts is found in Table E- 1: Distribution of Cattle Clones and Comparator Populations.

[120]Cyagra Inc. is a privately held biotechnology company commercializing SCNT technology for the agricultural sector.

Comparators were approximately age-matched animals reared on the same farms or facilities as clones. The comparators were not born at the same locations, and do not represent the same distribution among breeds as the clones. Comparator animals were not clones, but were produced by either artificial insemination (AI) or natural breeding, from either primiparous (heifers) or multiparous dams, and were all delivered vaginally. Blood samples from neonatal comparator animals were taken after colostrum administration, while neonatal clones were sampled prior to receiving colostrum.

These animals do not provide a strict biological comparator that has experienced the same treatments and conditions as the clones. For example, the culturing conditions in the embryonic phase for cloned embryos could be more closely compared with those encountered by animals generated by *in vitro* fertilization. These comparators are not, strictly speaking, "control" animals.

Further, given the approximate age- and breed-matching, this dataset should not be evaluated in the same manner as a tightly controlled prospective "laboratory" experiment. Rather, our opinion is that this dataset should be viewed as an attempt to compare health and laboratory test values between clones and conventional animals comprising part of national dairy and beef herds. These data were not generated or collected under "Good Laboratory Practices," and we have not attempted to audit the data except insofar as we have detected errors or requested clarification(s) from Cyagra.

Table E- 1: Distribution of Cattle Clones and Comparator Populations for Blood Analyses

	Number of Animals			
	neonates	**1 to 6 months**	**6 to 18 months**	**Totals**
Clones	10	46	18	67
Comparators	17	47	21	83

In Table E-1, 7 (of the 46) 1-6 month clones and 2 (of the 47) 1-6 month comparators were sampled in the neonatal group. The 1 to 6 month and 6 to 18 month cohort information was collected within a relatively short time frame. These data may best be thought of as a "snapshot" view of the animals during their development, rather than a longitudinal study in which the same animals are followed over some period of time. In fact, only nine animals were sampled or examined at more than one time point (at birth and weaning), and of those, seven were clones and two were controls (Clone ID# 71, 72, 73, 78, 79, 119, and 132; Control ID# 135 and 162).

1. Description of Clones

All clones were derived from actively dividing cells from skin biopsies; recipient oöcytes were obtained from commercial abattoirs. After 7 or 8 days of *in vitro* culture, morula or blastocyst stage reconstructed embryos were implanted into recipient Holstein heifers. Pregnancies were monitored closely, and with few exceptions, clones were delivered via Caesarean section (C-section) to reduce the risk associated with birth. Blood samples were drawn from the neonates prior to colostrum administration.

Table E-2 summarizes the information on samples taken from calves within the first 24 hours of birth. Some of the animals in the Cyagra dataset required some supportive care immediately after birth (*e.g.,* glucose, warming, or supplemental oxygen), and many (n=29 out 134) received umbilical surgery after birth. Enlarged umbilical vessels which do not close naturally after birth are an identified hazard for clone calves, and many of these calves received surgery to prevent complications such as umbilical infections and bleeding (see subsequent discussion on veterinary examinations and health status). This appears to be a fairly common problem in clones, and may be associated with poor placentation. However, no direct causal attribution can be made at this time to any particular developmental pathway causing the umbilical problems.

Health anomalies noted in surviving animals for which there are no additional follow-up data include diarrhea, fever, anemia, heart murmur, and slight contracture of the flexor tendons (referred to as "contracture").

Of the 134 clones in this review, 28 were stillborn, died, or were euthanized within 48 hours of birth, leaving 106 animals (or 79 percent) alive two days after birth. At the time that data were collected on these animals (late March 2003), 67 were alive (64 percent of those surviving to 48 hours, or 50 percent of those born or delivered). Eleven (10 percent) of the animals alive at 48 hours died within approximately one and a half years later. These data are summarized in Table E-2. Of the eleven deaths between 48 hours and one and a half years later, Cyagra considers two deaths not related to cloning, and the other nine as "related, possibly related, or questionably related" to cloning. Of those fitting the "related (to some degree) to cloning" category, one was clearly a fetal developmental anomaly: flexor tendon contracture ("contracture"); three experienced difficulties with the umbilicus ultimately leading to death either via infection or adhesions; two had gastrointestinal problems with bloat or adhesions; two had circulatory problems; and one animal was euthanized for "failure to thrive."

Table E-2: Summary of Outcomes for Clones Not Surviving Birth

Animal Number	Birth Weight (kg)	Age at Death (days)	Problems Noted	Cause of Death
3	NP[1]	0	Abnormal delivery	Stillborn
6	NP	16	NP	Accident; hung in stall
11	NP	0	Abnormal delivery	Euthanized
12	NP	0	Ruptured uterus in recipient	Stillborn
13	NP	0	NP	Unknown
14	33.2	13	Contracture[2], umbilical infection	Septicemia
16	50.0	2	Slack abdomen, umbilical problems, breathing difficulties	Failure to transition to neonatal circulation
18	68.2	0	Polycystic kidneys	Stillborn
19	69.1	0	Umbilical problems, flaccid abdomen	Stillborn
20	NP	0	Abnormal development	Euthanized
23	45.5	0	Abnormal development, internal bleeding, umbilical problems	Euthanized
28	NP	0	NP	Stillborn (C-section)
29	NP	0	Abnormal development	Euthanized
31	76.8	0	Abnormal renal development	Euthanized
34	NP	0	NP	Stillborn (C-section)
43	NP	1	Diarrhea	Rotavirus
47	NP	0	NP	Stillborn (C-section)
48	54.5	0	NP	Stillborn (C-section)
49	NP	0	NP	Stillborn (C-section)
51	NP	0	Flaccid abdomen, “bulldog”	Stillborn (C-section)
52	NP	0	NP	Stillborn (Fetotomy)
54	59.1	0	Reverted to fetal circulation, cardiac, neurological problems	Euthanized
57	NP	23	Ruptured abomasum	Ruptured abomasum
63	NP	60	Loss of hair, appetite, muscle	Euthanized/failure to thrive
65	61.4	3	Lethargic	GI transit; adhesions from umbilical bleeding
66	54.6	149	Contracted tendons, recurring bloat, large umbilicus requiring surgery	Bloat/GI motility problems
68	NP	0 (2 weeks	Pericarditis	Unable to determine

Table E-2: Summary of Outcomes for Clones Not Surviving Birth

Animal Number	Birth Weight (kg)	Age at Death (days)	Problems Noted	Cause of Death
		premature)		
77	NP	47	Umbilical problems	Severe contracture, unresponsive to therapy
80	NP	1	Diarrhea	Rotavirus
86	NP	0	Severe contracture, fluid filled belly	Euthanized.
92	NP	0	Depressed, pus in umbilicus	Unable to determine
95	NP	0	Severe contracture	Euthanized
97	NP	0	Severe contracture, fluid filled belly	Euthanized
105	45.5	0	Severe twisting of neck, contracture	Euthanized
107	NP	2	Hypoxemia, rapid deterioration	Euthanized
109	NP	0	Abnormal development	Euthanized
113	NP	22	Nephritis	Pyelonephritis[3]/ umbilical infection
123	NP	9	Contracted front fetlocks	Pyelonephritis/ umbilical infection
125	NP	0	Severe contracture, rotation	Euthanized

[1]NP = Not provided
[2]Contracture is a condition in which muscles have a fixed, high resistance to stretching due to fibrosis of the tissues supporting the muscles or joints, or from disorders of the muscle fibers.
[3]Pyelonephritis is an inflammation of the kidney due to bacterial infection.

2. Evaluations Performed

Several types of information including veterinary records, clinical chemistry measurements, hemograms,[121] and urinalysis are provided in this dataset. Not every collectable data point has been provided for each animal. Some information is unavailable because use of the data in a review such as this was not anticipated at the time the data were collected. In addition, dispersal of clones to their ultimate owners limited data collection to the degree to which owners made information or animals available. Nonetheless, this is the largest collection of information on the health status of non-transgenic clones of which we are aware, and the most detailed with respect to health status and laboratory tests.

[121] A hemogram is a panel of measurements characterizing the nature of the circulating blood in an animal or human.

The dataset includes information on the following:

- Breed from which donor cells were collected
- Gender of the donor
- Birth date of the clone
- Birth status (alive, stillborn)
- Birth weight
- Perinatal health status and veterinary/supportive care provided
- Health status of animals between two and twelve months of age
- Veterinary care, including treatment with drugs, surgery, or other therapeutic interventions
- Standard blood chemistry assays (Large Animal Panels)
- Assays for serum Insulin-like Growth Factor-1 (IGF-1), estradiol-17β, amylase, cholesterol, and bile acids
- Complete blood counts (CBC) and differentials
- Standard urinalysis

Comprehensive veterinary examinations were performed by licensed cattle veterinarians. Blood samples were drawn within a few hours of birth, or at the time of veterinary examination. For CBC, blood was collected into standard EDTA-treated collection tubes; additionally, two unstained and unfixed air-dried smears were provided. For chemical analyses, whole blood was collected, allowed to clot, and the serum fraction separated by centrifugation. Laboratory analyses were all performed at the Cornell University's Animal Health Diagnostic Laboratory.

3. CVM's Analysis of Cyagra Data: Method

Our goal in evaluating the Cyagra dataset has been to determine whether extensive interrogation of the health status of the clones, including clinical chemistry and hematology, could

(a) Distinguish clones from comparators;
(b) Determine whether the health status of the clones was inferior to conventional animals and offer a predictor of a successful outcome; and
(c) Determine whether any of the information indicated concerns regarding animal health or food safety.

We note that this was not a "blinded" analysis of the provided data. No attempt was made to disguise the identity of the animals, and whether they were clones or comparators. CVM personnel engaged in performing the evaluation included veterinarians, animal scientists,

toxicologists, and risk assessors, with extensive training in evaluating clinical and physiological measurements of animals traditionally consumed as food in the US.

For the overall health status of animals, the veterinary records were reviewed for notations indicating therapeutic interventions (including administration of colostrum, vaccines, dehorning, surgeries, drug therapies, etc.). Clinical and hematologic data were compared to both reference ranges provided by the testing laboratory and to the comparator animals. Additionally, laboratory values from the comparator animals were also compared to the testing laboratory to determine the degree to which the comparator group was represented by the testing laboratory's reference range (see Results). In general, urinalysis data were only used qualitatively as confirmation of outcomes noted in the clinical chemistry (*e.g.,* glucose, BUN or creatinine levels). Table E-3 provides a summary of the analyses performed and tabulated in the Charts indicated.

Outcomes were reviewed on an analyte basis across a cohort of animals (analyte evaluation), and on a per animal basis across analytes (animal evaluation). The questions asked for each animal and analyte tested were "*How many of the total animals tested exhibited values outside the comparator/testing laboratory reference range for Analyte X*?" and "*How many values outside the comparator/testing laboratory reference range does Animal Y exhibit?*"

Table E-3. Summary of Charts Describing Comparisons

	Clones: Reference Range	**Clones: Comparators**	**Comparators: Reference Range**
Clinical Chemistry: 6 to 18 months 1 to 6 months neonates	Chart 300 Chart 200 Chart 100	Chart 301 Chart 201 Chart 101	Chart 302 Chart 202 Chart 102
Hematology: 6 to 18 months 1 to 6 months neonates	Chart 310 Chart 210 Chart 110	Chart 311 Chart 211 Chart 111	Chart 312 Chart 212 Chart 112

The Charts are a graphical summary of CVM's analyses. For each chart, the unique identification number associated with each animal ("ID#" or "animal number") is listed in columns horizontally across the top of the table; the analysis performed is listed in rows vertically down the side of the table. If the value being evaluated fit within the comparison range being used for that interrogation, a black rectangle was recorded in the cell corresponding to the animal column/analyte row pair (■). If the value was outside the comparator range, but judged to be not clinically relevant, a gray rectangle (▢) was recorded. If the value recorded was above or below the clinically relevant range, an arrow indicating whether the value was greater or less than the

range was inserted (↑↓). Values that were considered to be so far out of range as to be physiologically incompatible with a healthy animal but unsupported by related clinical measurements were deemed artifact and labeled "X." For example, a calf with a blood glucose level of 4 mg/dl would be comatose or dead. If the sample came from an animal that was not comatose or in distress, and there were no other related clinical measurements normally associated with abnormal blood glucose, we assumed that the measurement was an artifact. Missing values were represented by an asterisk (*).

4. CVM's Analysis of Cyagra Data: Results

a. Comprehensive Veterinary Examinations

Comprehensive Veterinary examinations were performed on 53 clones and 2 non-clones, and included explicit evaluations of the following:

- Demeanor
- Posture
- Gait
- Body Condition
- Skin and coat
- Vocalization
- Lungs (Auscultation)
- Nerves
- Integument
- Musculo-skeletal system
- Cardiovascular system
- Oral/Pharyngeal region
- Urine
- Gastrointestinal system
- Genitals
- Neurological examination
- Peripheral lymph nodes
- Responsiveness of pupils to light
- Corneas and eyelids
- Umbilicus
- Weight
- Heart Rate
- Respiration rate
- Temperature
- Feces

The calves in this study were examined by veterinarians specializing in cattle at roughly 1-6 months of age or at 6-18 months of age. The most consistent abnormality reported for clones was umbilical surgery, often described as umbilical hernia surgery. In some instances, the records stated, "umbilicus – had surgery." Some other comments on the umbilicus were: "had umbilical hernia surgery," "ventral hernia," and "1 ½" hernia," "fluid filled mass," "umb. stump." In the initial submission of 58 animals, 26 animals had umbilical surgery. Other abnormalities reported included two clones with musculo-skeletal abnormalities, one with slight precocious (early) mammary development, two with harsh lung sounds, three cryptorchid (undescended testicles)

bull calves, and one with premature ventricular contractions (PVCs, a form of cardiac arrhythmia) every 5 – 10 heartbeats.

The two clones with musculo-skeletal abnormalities included a Holstein heifer (ID# 79) with thick withers, enlarged left carpus, and leg that deviated laterally, and an Angus heifer that was a dwarf tending to gastro-intestinal bloat (Clone #108). These are obvious abnormalities and the animals were culled. The calf with slight mammary development was a 4½ month old Jersey (Clone #87). This age is young for mammary development but the phenomenon sometimes occurs in conventional heifers if they are overfed. There is no notation of follow up to determine if the calf continued to develop precociously.

The two clones with harsh lung sounds were a Holstein heifer (Clone # 41) and an Angus heifer (Clone #58). Both also had umbilical surgery. A note at the bottom of the Angus heifer's exam sheet stated that the heifer "may not return home due to permanent lung damage." There is no indication as to whether this animal was culled. Three Holstein bull clones derived from the same cell line were diagnosed with a retained testicle (cryptorchid) (Clones #128, 130, 131). Although cryptorchidism is not common in bull calves, it is thought to be heritable and is seen with some regularity. Bulls exhibiting cryptorchidism would fail their breeding soundness exams, and would not be used for breeding,[122] but would not be refused by an inspector at slaughter.

A Holstein bull calf clone (Clone #126) was diagnosed with premature ventricular contractions from a single exam, but no subsequent follow up is provided to determine whether the animal outgrew the condition or whether the animal was culled. The frequency of cardiac arrhythmias in conventional calves is unknown. Thoracic auscultation (listening to the chest with a stethoscope) or more elaborate procedures are needed to detect cardiac arrhythmias. Calves are rarely examined with thoracic auscultation unless they show signs of illness.

b. Conclusions from Veterinary Examinations

The adverse physical exam findings noted in this limited sample of clones do not present a food safety issue for several reasons. One of the precepts of this risk assessment is that animals found to have a disease or condition that would render them adulterated (e.g., unfit for food, unhealthful, unwholesome) are excluded from the food supply, as normally happens with conventional animals. Dwarf animals from conventional breeding would likely be culled

[122] Cryptorchidism is undesirable because of its heritability, its adverse effect on fertility, and potential for the development of testicular cancer in animals living long enough to allow neoplasia to develop. From a veterinary standpoint, however, testicular neoplasia is more of an issue with companion animals, as they are generally longer-lived than farm animals.

depending on the extent of the physical abnormality. Pre-pubescent mammary development, lung sounds, cryptorchidism, and cardiac arrhythmias are not conditions that typically exclude animals from food use. If the disease process had progressed to an extent sufficiently severe to cause systemic changes (*e.g.,* liver congestion, enlarged heart, edematous lungs), the carcass would be condemned on inspection at the slaughtering plant. In fact, all of these conditions occur in conventional animals.

With respect to animal safety, these conditions may pose some cause for concern. Our review of these data indicates that the clone cohort appears to exhibit a higher incidence of abnormalities than might be expected in a random sample of conventional calves. There is, however, an absence of data on the prevalence of these outcomes in contemporary cattle. As some of these defects (*e.g.,* dwarfism, cryptorchidism) likely have a hereditary component, in the absence of information on the donor cattle and their individual histories, we cannot determine whether the defects result from the cloning process, the selection of the donor nucleus, or some combination of those factors. The clustering of cryptorchidism in clones from one cell line, for example, implies that heredity may indeed be a contributing factor in the appearance of that outcome. Comparison with datasets on animal health from other clone producers would be instructive in determining whether these health problems are common among clones generated by different methods and multiple cell lines.

c. Laboratory Values: Selection of Most Appropriate Comparator

Two comparators were available for evaluating the Clones: the Cornell Animal Health Diagnostic Laboratory ("Reference Range") and approximately age-matched, and breed-distributed cohort of animals contemporarily reared at the same farms as the clones ("Comparator Population" or "Comparators"). The Reference Range population from the Cornell Laboratory is described as follows:

> "*We establish reference intervals by collecting blood from at least 50* ***adult healthy*** *animals. These healthy animals are obtained from a variety of sources (e.g., student- or faculty-owned). Therefore, our reference intervals are only applicable for adult animals and not young animals. Results from young animals may fall outside our reference intervals because of age-dependent changes in their analytes. For example, phosphate concentrations and alkaline phosphatase activity are higher in young animals and decrease to within reference intervals at about one year of age."* (http://www.diaglab.vet.cornell.edu/clinpath/reference/)

Follow-up conversation with the laboratory indicates that the animals used to establish the laboratory's reference range were exclusively dairy cows, and thus do not represent the beef

breeds that are included in the Cyagra clone cohort or comparator cohorts, and may not include bulls. In addition, it is important to remember that the reference range is selected as a statistical distribution containing about 95 percent of the normal samples. As a result, as many as 5 percent of the test values will likely fall outside that range. Statistically, when numerous tests are run on the same animal, the chance of obtaining one or more results outside the "normal range" rises based on chance alone and not a disease state.

Table E-3a: Fraction of Blood Values Within Comparison Range

Animals	Analysis	Clones: Cornell Reference Range		Clones: Comparator Population		Comparator Population: Cornell Reference Range	
		Chart	FCWR	Chart	FCWR	Chart	FCWR
Peripubertals (6-18 months)	Clinical Chemistry	300	**0.75**	301	**0.99**	302	**0.73**
	Hematology	310	**0.73**	311	**0.99**	312	**0.71**
Juveniles 1-6 months)	Clinical Chemistry	200	**0.62**	201	**0.96**	202	**0.71**
	Hematology	210	**0.59**	211	**0.96**	212	**0.61**
Neonates (<48 hours)	Clinical Chemistry	100	**0.31**	101	**0.90**	102	**0.36**
	Hematology	110	**0.61**	111	**0.90**	112	**0.62**

FWCR= Fraction contained within range of comparison, calculated by determining the number of out of range analytes of potential clinical relevance to the total number of measurements collected in each Chart.

Table E-3a provides a summary of the Charts evaluating the clinical chemistry and hematology tests performed on the Cyagra clones compared with the comparator population, and the Cornell Reference Range. In addition, the comparator population was compared to the Cornell Reference Range. First, as cautioned by the Cornell Laboratory, the Reference Range is not a good comparator for young animals. A number of the clones and comparators fall outside the Reference Range[123] but the similarity to the Reference Range increases with age for both clone and comparator populations. Approximately half the animals in the older cohort were less than one year of age, however, and all clones and comparators were less than two years of age. All of the animals in the older cohorts were still growing and thus do not match the laboratory reference adult cattle population well. Clearly, then the most relevant comparison for the clone cohorts in this review is the comparator population.

d. Conclusions Regarding Clone and Comparator Population Cohorts in Aggregate

[123] It should be noted that because the reference range represents only 95 percent of the animals used in its derivation, even comparison of the animals used for the derivation will not fit exactly within the distribution. Thus, if the reference range were expanded to include those values outside the 95 percent distribution, it is likely that the clone and comparator populations would show a higher degree of "fit" than is observed in this analysis.

Review of the degree to which the clone cohorts have laboratory values that fit within those of the comparator population cohorts indicates the following:

1. Even at birth, 90 percent (107 of 119 measurements) of the hematology values, and 90 percent (272 of 324 values) of the clinical chemistry values lie within the values of the comparator population (Table E-3, Charts E101 and E111). This is particularly instructive, considering that many of the clones required some assistance immediately after birth (no similar records were kept for the comparators, but we assume that no extraordinary measures were taken, and were informed that all comparators were born vaginally). Further, clones had blood samples drawn before colostrum administration, while the comparators had blood samples drawn after colostrum administration, but within 24 hours after birth. Colostrum consumption (quantity and quality) influences certain laboratory values (*e.g.,* globulin, total protein, GGT).

2. The 1 to 6 month age cohorts are even more similar to each other than the neonatal cohorts: both the clinical chemistry and hematology values have 96 percent and 95 percent concordance respectively (707 of 742 of the hematology measurements and 1,404 of 1,462 of the clinical chemistry measurements for clones are within the clinically relevant ranges) (Charts 201 and 211).

3. The 6 to 18 month cohorts are almost superimposable with respect to laboratory values (Charts 301 and 311). Only three of the 294 hematological values and seven of the 592 clinical chemistry measurements were outside the clinically relevant ranges, significantly less than would be expected by chance alone.

Based on clinical chemistry and hematology values, it is not possible to distinguish between these two cohorts (clones and comparators). The superimposability of the laboratory values and the absence of any significant health observations in the clones (based on the limited number of explicit veterinary exams) leads to the conclusion that the health of these animal clones during the 6-18 month period is not inferior to that of conventional animals.

Because we have concluded that the comparator group is the appropriate basis for comparison for the clones, all subsequent discussion regarding clinical and hematological values will be considered in that context.

e. Animal and Analyte Specific Analyses

In addition to evaluating the overall status of the clone and comparator cohorts, individual animal and analyte data were reviewed to determine if more detailed evaluations could provide either confirmation of the overall health of the animals, or to serve as indicators of potential health problems that might be present in the animals that were not detected on the comprehensive veterinary examinations. For each Chart, the following two questions were asked:

1. "For *all* of the clones in this age cohort, how many of the values for each analyte were out of the range established by the comparators? (*i.e.,* looking across each row, how many arrows or grey rectangles were present?), and

2. "For each clone in this cohort, how many of the analytes were out of the range established by the comparators?" (*i.e.,* looking down each column of the Chart).

There are three overall issues addressed by this evaluation:

1. Whether the laboratory values of the clones were similar to those of the comparator population on an animal-by-animal level, or whether it would be possible to distinguish between the two populations based on the clinical chemistry and hematology data. A finding of similar laboratory values would provide confidence that there were no material differences in metabolic, immunologic, and hematopoetic (blood producing) functions between clones and conventional animals;

2. Whether the clones respond to the internal (growth and maturation) and normal external (stressors, disease) environments appropriately (analyte based approach); and

3. Whether the individual values can be used to predict the long-term viability of that animal or that cohort (analyte and animal approaches combined).

A description of the parameters that were evaluated and their relation to physiological status is provided in Appendix F: The Comprehensive Veterinary Examination.

Clinical chemistry and hematology responses are best evaluated in the context of the whole animal, including its age, species, breed, husbandry, geographic location, reproductive status, and the laboratory performing the analysis. Laboratory findings complement the subjective physical diagnosis of the patient by providing objective information for the process of differential diagnosis, monitoring treatment, and formulation of a prognosis (see Appendix