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Form Approved Through 05/2004 | OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Application

Do not exceed character length restrictions indicated.

LEAVE BLANK—FOR PHS USE ONLY.		
Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*
cGMP Recombinant FIX for IV and Oral Hemophilia B Therapy

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☐ NO ☒ YES
(If "Yes," state number and title)
Number: PAR-04-023 Title: Bioengineering Research Partnerships

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR | **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME *(Last, first, middle)*
Velander, William H.

3b. DEGREE(S)
Ph.D.

3c. POSITION TITLE
Professor and Chair

3d. MAILING ADDRESS *(Street, city, state, zip code)*
207 Othmer Hall
Lincoln, NE 68588-0642

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Chemical Engineering

3f. MAJOR SUBDIVISION
College of Engineering and Technology

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: 402-472-3697 FAX: 402-472-6989

E-MAIL ADDRESS: wvelander2@unl.edu

4. HUMAN SUBJECTS RESEARCH
☒ No
☐ Yes

4a. Research Exempt ☐ No ☐ Yes
If "Yes," Exemption No.

4b. Human Subjects Assurance No.

4c. NIH-defined Phase III Clinical Trial ☐ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☒ Yes

5a. If "Yes," IACUC approval Date
pending

5b. Animal welfare assurance no.
A3459-01PHS

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
01-01-05	12-31-09	$1,372,741	$1,996,007	$6,847,010	$9,955,598

9. APPLICANT ORGANIZATION
Name University of Nebraska-Lincoln
Address Pre-Award Development
312 N. 14th Street
Alexander West
Lincoln, NE 68588-0430

Institutional Profile File Number (if known)

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☒ State ☐ Local
Private: → ☐ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
1470491233A3
DUNS NO. 55-545-6995
Congressional District 1

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name Norman O. Braaten
Title Director, Pre-Award Development
Address 312 N. 14th Street
Alexander West
Lincoln, NE 68588-0430
Tel: 402-472-3171 FAX: 402-472-9323
E-Mail: nbraaten1@unl.edu

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Norman O. Braaten
Title Director, Pre-Award Development
Address 312 N. 14th Street
Alexander West
Lincoln, NE 68588-0430
Tel: 402-472-3171 FAX: 402-472-9323
E-Mail: nbraaten1@unl.edu

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a.
(In ink. "Per" signature not acceptable.)

DATE

15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13.
(In ink. "Per" signature not acceptable.)

DATE

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Hemophilia B is a bleeding disorder resulting from congenital deficiency of coagulation protein Factor IX. Without optimal therapy, patients suffer crippling debilitations, chronic pain, and cognitive impairment. Optimal therapy - frequent dosing with Factor IX to reduce bleeding episode frequency - is currently available to only a small fraction of U.S. patients, largely because of cost and limited supply; 80% of the world's hemophilia patients receive no therapy. The long-term goal of this research is development of an abundant, pure, safe, and effective therapy for the global hemophilia patient population using recombinant human coagulation proteins produced in the milk of transgenic pigs. This proposal describes studies that are based upon innovative bioengineering technologies to refine and scale-up production of clinical grade Factor IX, characterize product formulated for intravenous dosage, complete animal studies required for clinical trials, and ultimately develop an oral dosage form to provide optimal therapy for U.S. hemophilia B patients. This approach capitalizes on existing genetically engineered transgenic pigs that synthesize up to 1000 IU/ml (4 g/L) of human Factor IX in milk and preliminary evidence for favorable hemostatic and pharmacokinetic properties of this purified Factor IX in a hemophilia B mouse model. A high probability of success is expected because of the soundness and state of development of the basic research already completed and the breadth and complimentarity of the strengths of the team. Dr. J. Cooper of ProGenetics LLC will supply FDA- and USDA-compliant Factor IX milk. At the Univ. Nebraska-Lincoln, Dr. M. Meagher provides expertise in bioprocess engineering and current Good Manufacturing Practices production of recombinant proteins; Drs. W. Velander and K. Van Cott contribute expertise in transgenic animal engineering and characterization of human Factor IX. Dr. M. Manning of Colorado State University brings expertise in the formulation of stabilized liquid and lyophilized proteins for oral and injectable dosing. Dr. S. Abramson of LifeSci Partners has two decades of biopharmaceutical development experience encompassing clinical, technical, and regulatory expertise, including AlphaNine®SD (purified plasma-derived Factor IX). Drs. P. Monahan and T. Nichols of UNC-Chapel Hill have unique preclinical experience with hemophilia B mice and dogs that will be used to test the safety and efficacy of the Factor IX products.

PERFORMANCE SITE(S) *(organization, city, state)*

University of Nebraska-Lincoln, Lincoln, NE
ProGenetics LLC, Blacksburg, VA
University of North Carolina-Chapel Hill, Chapel Hill, NC
Colorado State University, Fort Collins, CO
LifeSci Partners, Altadena, CA

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below.
Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Velander, William, Ph.D.	University of Nebraska-Lincoln	Principal Investigator
Abramson, Stephan, Ph.D.	LifeSci Partners	Lead Investigator
Cooper, Julian, Ph.D.	ProGenetics LLC	Lead Investigator
Dernell, William, D.V.M, M.S.	Colorado State University	Key Person
Manning, Mark, Ph.D.	Colorado State University	Lead Investigator
Meagher, Michael, Ph.D.	University of Nebraska-Lincoln	Lead Investigator
Monahan, Paul, M.D.	University of North Carolina	Lead Investigator
Nichols, Timothy, M.D.	University of North Carolina	Lead Investigator
Swanson, Todd, Ph.D.	University of Nebraska-Lincoln	Key Person
Van Cott, Kevin, Ph.D.	University of Nebraska-Lincoln	Lead Investigator

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ Yes ☐ No

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Summary Budget--Direct Costs

UNL Activities						
Analytical Methods and Characterization	$308,076	$214,970	$184,611	$183,584	$183,166	**$1,074,407**
Purification	0	186,350	176,250	75,120	0	**437,720**
cGMP Production	45,000	34,500	0	267,885	225,741	**573,126**
Quality Assurance	156,048	160,729	165,551	170,518	175,633	**828,479**
Quality Control	210,648	275,385	248,621	211,738	205,319	**1,151,711**
UNL Project Management	226,134	232,918	239,906	247,103	254,516	**1,200,577**
UNL Subtotal	***$945,906***	***$1,104,852***	***$1,014,939***	***$1,155,948***	***$1,044,375***	**$5,266,020**
Subawards						
Colorado State Univ.--M. Manning, W. Dernell						
Formulation--direct costs	163,318	178,205	0	0	0	**341,523**
UNC-Chapel Hill						
P. Monahan						
Mouse Model Experiments--direct costs	51,507	0	55,114	56,844	0	**163,465**
T. Nichols						
Dog Trials--direct costs	0	0	125,458	0	163,994	**289,452**
LifeSci Partners						
Clinical trial development--direct costs	86,150	88,735	91,397	94,138	96,963	**457,383**
Progenetics LLC						
Provide milk--direct costs	125,860	0	65,776	67,749	69,782	**329,167**
Subawards Subtotal	426,835	266,940	337,745	218,731	330,739	**1,580,990**
Yearly Totals	**$1,372,741**	**$1,371,792**	**$1,352,684**	**$1,374,679**	**$1,375,114**	**$6,847,010**

Analytical Methods and Characterization (UNL)

Principal Investigator/Program Director (Last, first, middle): Velander, William H.

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY

FROM 1/1/2005 THROUGH 12/31/2005

Personnel *(Applicant organization only)*					DOLLAR AMOUNT REQUESTED *(omit cents)*		
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Kevin Van Cott	co-PI	9	33%	85,490	$28,212	$7,335	$35,547
S. Todd Swanson	co-PI	12	10%	71,585	$7,159	$1,861	$9,020
TBN Research Technologist II	Scientist	12	90%	50,000	$45,000	$11,700	$56,700
TBN Research Technologist I	Scientist	12	90%	45,000	$40,500	$10,530	$51,030
TBN Reesearch Technologist I	Scientist	12	90%	45,000	$40,500	$10,530	$51,030
							$0
				SUBTOTALS	$161,370	$41,956	$203,326

CONSULTANT COSTS Outside Contract Services - N-terminal sequencing, amino acid analysis, Gla analysis, Maintenance contracts — $11,250

EQUIPMENT *(Itemize)*

Beckman ALC Advance Coagulation Analyzer — $50,000

SUPPLIES *(Itemize by category)*

	Chormatographic	(Capillary & slab) Electrophoret	Immunoassay	APTT and Chromogenic	Peptide Maps	Spectroscopic and Fluor. tests
Columns	6,000	500	0	0	3,000	0
Reagents	6,000	2,000	3,000	2,000	1,000	500
Disposables	2,000	1,000	1,000	250	500	500
Gels	0	1,500	0	0	0	0
Standards	0	1,000	1,250	3,000	500	500
Kits	0	1,000	0	0	0	0

General lab supplies--lab notebooks, labeling tape, toner cartriges, media, 2,500 — $40,500

TRAVEL
ASH or ACS meeting, one person — 2,000

PATIENT CARE COST	INPATIENT	
	OUTPATIENT	

ALTERATIONS AND RENOVATIONS (Itemize by category)

OTHER EXPENSES (Itemize by category)
Publications costs, 500, overnight shipping for samples and documents, 500 — $1,000

SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD — $308,076

CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	
	FACILITIES AND ADMINISTRATIVE COSTS	

TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)* — **$308,076**

SBIR/STTR Only: FEE REQUESTED

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

Analytical Methods and Characterization (UNL)

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		203,326	152,220	138,109	142,252	142,252
CONSULTANT COSTS		11,250	16,875	11,250	11,250	11,250
EQUIPMENT		50,000	0	0	0	0
SUPPLIES		40,500	42,550	31,850	26,600	26,350
TRAVEL		2,000	2,060	2,122	2,185	2,251
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		1,000	1,265	1,280	1,296	1,063
SUBTOTAL DIRECT COSTS		308,076	214,970	184,611	183,584	183,166
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	F&A					
TOTAL DIRECT COSTS		308,076	214,970	184,611	183,584	183,166
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$1,074,407
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Propposed Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Analytical Methods Development will begin in Year 1 and have the following tasks:
1. Characterize the structure and function of transgenic Factor IX
2. Generate SOPs that will support analysis of samples from Process Development, Quality Control, and cGMP production of transgenic Factor IX.

As such, the general plan for Methods Development research is as follows
• Year 1 – Develop Characterization assays and help generate SOPs.
• Year 2 – Perform in-process assays to support Process Development and develop lot-release assays and help generate SOPs.
• Years 3-5 – Perform routine characterization, in-process, lot release, and stability assays to support Process Development and cGMP production.

UNL Analytical Methods and Characterization, Budget Justification, continued

PERSONNEL

Kevin E. Van Cott, Ph.D., Lead Investigator
Dr. Van Cott will oversee the development of Factor IX-specific analytical methods that are required for molecular structure characterization, analysis of purity, and determination of activity and stability. He will work with Dr. Swanson to coordinate in-house operations with out-sourcing activities, review project development reports and monthly reports, teleconference with other Lead Investigators, and interface with Dr. Meagher and the Quality Control, Quality Assurance, Process Development, and cGMP Manufacturing groups. Dr. Van Cott will spend 35% of his time on this project, and a salary is requested for the duration of this project.

S. Todd Swanson, Ph.D., Key Person
Dr. Swanson will oversee the development of general protein chemistry analytical methods that are used to characterize Factor IX structure and coordinate out-sourcing activities (e.g., amino acid analysis, N-terminal sequencing). He will spend 10% of his time on this component of the project in Year 1, 5% in Year 2, and then he will transition to Quality Control efforts. A salary is requested for his efforts.

TBN, Research Technologists
Three scientists will be required to develop the analytical methods for this project. Two Research Technologist I and one Research Technologist II scientists will jointly contribute 270% of their time in Year 1. We foresee that one Research Technologist I scientist will be responsible for Factor IX-specific assays – aPTT, chromogenic, phosphorylation, and SDS-PAGE). One Research Technologist I scientist will be responsible for development of carbohydrate analyses and isoelectric focusing (both using capillary electrophoresis). One Research Technologist II scientist will be responsible for more general protein chemistry assays – HPLC methods, peptide mapping, gamma-carboxyglutamate content, amino acid analysis, and N-terminal sequencing. SOPs for these methods will be generated during Year 1. In Year 3, one of the Research Technologists will transition to Quality Control.

EQUIPMENT

We are requesting $25,000 toward the purchase of a Beckman ACL-100 automated coagulation analyzer for aPTT assays. The aPTT assay is notorious for operator-dependent variability, and an automated analyzer (as opposed to a manual coagulometer) will simplify writing of the SOP and reproducibility of the results.

CONTRACT SERVICES

1. University of Nebraska Medical Center – Protein Structure Core Facility offers quality services at our institutional rate
    a. Protein Sequencing Services – 5 N-terminal sequences @ $350/sample ($1,750)
    b. Amino acid analysis (acid hydrolysis) – 5 samples @ $100/sample - $500
2. Commonwealth Biotechnologies (Richmond, VA)
    a. gamma-carboxyglutamate (Gla) analysis by basic amino acid hydrolysis – 10 samples @ $200/sample - $2,000)
3. HPLC Contract maintenance ($7,000) – covers maintenance contract with manufacturer's service technician: preventative maintenance to keep machines operating and guarantees rapid repair if necessary. Two HPLCs will be maintained for this aspect of the project.

SUPPLIES COSTS

Columns:

1. <u>Chromatographic:</u> HPLC analysis of Factor IX purity and concentration – two Vydac C4 reverse phase columns ($445/ea), two Vydac C8 reverse phase columns ($445/ea), two Vydac C18 reverse phase columns ($445/ea). MonoP and MonoQ high performance ion exchange columns ($1,000/ea). Corresponding guard columns kits ($160/ea) and refill cartridges ($150/pk).

2. Electrophoretic: Fused silica capillary columns for isoelectric focusing (cIEF) analysis and carbohydrate analysis by capillary electrophoresis
3. Peptide Mapping: one Vydac C4 reverse phase columns ($445/ea), one Vydac C8 reverse phase columns ($445/ea), one Vydac C18 reverse phase columns ($445/ea). Corresponding guard columns kits ($160/ea) and refill cartridges ($150/pk).

Reagents:

1. Chromatographic – high purity solvents, buffers and salts, ion pairing agents, and Millipore water polishing cartridges
2. Electrophoretic
    a. sample buffers, running buffers for SDS PAGE ($30/liter)
    b. reagents for carbohydrate analysis - fluorescent labeling reagents ($134/10mg).
3. Immunoassays
    a. Primary and secondary antibodies needed for sandwich ELISAs ($50-100/mg)
    b. Colorigenic reagents for ELISA plate development ($160/50 assays)
    c. Buffers and salts
    d. Protein standards for ELISAs (porcine immunoglobulins and albumin)
    e. PVDF membrane and colorigenic reagents for western blot development
4. aPTT and Chromogenic FIX assays - aPTT reagent ($60/20ml), Factor IX-deficient plasma ($100/6ml), Calcium chloride ($56/10ml), Owrens Buffer ($33/100ml), Imidazole buffer ($41/120ml); Normal Reference plasma ($125/10ml); Factor XIa ($50/100micrograms); Chromogenic substrate
5. Peptide Mapping - High-purity proteomics-grade trypsin ($60/50mg), Factor XIa ($50/100micrograms), and other proteases required to proteolyze Factor IX (e.g., *Staph. aureus* protease - $250/5mg); High purity solvents and ion pair agents
6. Spectroscopic and Fluorescent tests
    a. Calcium-induced fluorescent quenching assays for Factor IX – Factor IX standards

Disposables:

1. Chromatographic – fraction collection tubes, tubing, fittings, inline filters.
2. Electrophoretic
    a. SDS PAGE – pipet tips, gel loading tips, filter paper
    b. Carbohydrate analysis – autosampler vials, washers and fittings, pipet tips; vacuum-sealed vials (for hydrolysis) and crimper
4. Immunoassays - 96-well microtiter plates ($170/case of 60); pipet tips; reagent boats; microcentrifuge tubes
5. aPTT and Chromogenic FIX assays – 96-well microtiter plates; pipet tips; microcentrifuge tubes
6. Peptide Mapping – autosampler and fraction collection vials; pipet tips

Gels:

1. Electrophoretic – Precast gels (Invitrogen, $10/gel) for SDS PAGE and western blots; 2D gels and IPG strips.

Standards:

1. Electrophoretic
    a. SDS PAGE – MW Standards
    b. Carbohydrate analysis – monosaccharide and oligosaccharide standards
2. Immunoassays – porcine albumin and immunoglobulin standards (IgG, IgA, IgM)
3. aPTT and Chromogenic FIX assays – FIX standards BeneFIX™ ($1600) and Mononine® ($1600); Factor IXa standard ($260/0.5mg)
4. Peptide Mapping – BeneFIX™ and Mononine®
5. Spectroscopic and Fluor tests - BeneFIX™ and Mononine®

Kits:

1. Electrophoretic – oligosaccharide profiling kit (contains standards, capillaries, buffers).

General Supplies: Funds are requested for official lab notebooks, toner cartridges for printing reports and data, sample labeling tape (resistant to temperature change), media for data backup (CD-RW disks).

Other Notes:

1. LAL assay development will be incorporated in Year 2.

Purification (UNL)

Principal Investigator/Program Director (Last, first, middle): Velander, William H.

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY					FROM 1/1/2006		THROUGH 12/31/2006
Personnel *(Applicant organization only)*					DOLLAR AMOUNT REQUESTED *(omit cents)*		
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Rick Barent	PDL Manager	12	30%	55,000	$16,500	$4,290	$20,790
TBN Research Tech I	Scientist	12	90%	45,000	$40,500	$10,530	$51,030
TBN Research Tech I	Scientist	12	90%	45,000	$40,500	$10,530	$51,030
				SUBTOTALS	$97,500	$25,350	$122,850

Item	Amount
CONSULTANT COSTS	$0
EQUIPMENT *(Itemize)*	$0
SUPPLIES *(Itemize by category)* Membranes for Cross flow microfiltration and ultrafiltration studies (bench and pilot scale), 15,000 Chromatography Resins for Process Development, 10,000 General Laboratory Supplies (reagents, SDS-PAGE Gels, columns, resins, etc.), 18,000 Service Agrements on BioCads and Maintanence of PDL equipment, 15,000	58,000
TRAVEL AIChE Annual Meeting--2 people	4000
PATIENT CARE COST — INPATIENT	
PATIENT CARE COST — OUTPATIENT	
ALTERATIONS AND RENOVATIONS (Itemize by category)	
OTHER EXPENSES (Itemize by category) Overnight Shipping to Colorado State Univ. (10 lb dry ice with samples) $50/shipment- 30 shipments	1,500
SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD	$186,350
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*	**$186,350**

SBIR/STTR Only: FEE REQUESTED

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

Purification (UNL)

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		0	122,850	115,630	37,126	0
CONSULTANT COSTS		0	0	0	0	
EQUIPMENT		0	0	0	0	0
SUPPLIES		0	58,000	55,000	33,500	0
TRAVEL		0	4,000	4,120	4,244	0
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		0	1,500	1,500	250	0
SUBTOTAL DIRECT COSTS		0	186,350	176,250	75,120	0
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	F&A					
TOTAL DIRECT COSTS		0	186,350	176,250	75,120	0
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$437,720
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Propposed Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

PERSONNEL

Rick Barent, M.S., Scientist

Mr. Barent will spend 30% of his time on the project managing the research technologists that will be doing the bench-scale research. He will have the responsibility of assisting in planning experiments, collection of data and electronic archiving, interpretation of data, report writing, overseeing the Purification Development Laboratory's (PDL) responsibility on writing the Production Batch Records, and will be involved in running experiments. Mr. Barent also will oversee the non-cGMP production of Factor IX in the PDL.

UNL Purification, budget justification, continued

TBN, Two Research Technologists I
These scientists will be responsible for running recovery and purification experiments. One Research Technologist I will focus a majority of his/her efforts on recovery methods, while the second Research Technologist I will focus on purification/polishing steps leading to a highly purified Factor IX suitable for clinical testing. The two research technologists will also be responsible for producing Factor IX under non-cGMP conditions using the Production Batch Records.

SUPPLIES COSTS
Membrane for Crossflow Studies: Membrane modules for pilot-scale studies (2 to 20 ft^2) that range in cost from $1,000 to $3,000 each will be needed for both microfiltration and ultrafiltration studies. The membranes will be used primarily for recovery studies, but also for concentration and buffer exchange of Factor IX. New membranes will be required for the non-cGMP production runs.

General Laboratory Supplies: This category is to cover the costs of reagents, such as chemicals, dry goods, electrophoresis gels and reagents, packed columns, individual membrane modules for small studies (0.05 to 0.5 ft^2), tubing, empty columns, etc. In order to balance the budget in accordance with the requirements of the RFA, a small amount of general laboratory supplies ($5,000) originally scheduled to be purchased in year 4 will be purchased in year 3.

Chromatography Resins: Five to ten liters of chromatography resin are necessary for the process scale-up studies and non-cGMP demonstration runs.

Service Agreement and Maintenance Costs: The BioCads are on service/maintenance agreements. Resources are necessary to maintain the pilot-scale equipment, such as the K'40 chromatography skid and the Model 50 membrane system. Such equipment must be maintained on a regular basis with o-rings and critical parts being replaced on a regularly.

cGMP Production UNL

Principal Investigator/Program Director (Last, first, middle): Velander, William H.

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY					FROM 1/1/2007		THROUGH 12/31/2007
Personnel *(Applicant organization only)*					DOLLAR AMOUNT REQUESTED *(omit cents)*		
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Scott Johnson	cGMP Coordinator	12	50%	75,945	$37,973	$9,873	$47,845
TBN Research Tech I-FFP	Operator	12	40%	47,600	$19,040	$4,950	$23,990
TBN Research Tech I-FFP	Operator	12	40%	47,600	$19,040	$4,950	$23,990
TBN Research Tech I-FFP	Operator	12	40%	47,600	$19,040	$4,950	$23,990
TBN Research Tech I-PPP	Operator	12	40%	47,600	$19,040	$4,950	$23,990
TBN Research Tech I-PPP	Operator	12	40%	47,600	$19,040	$4,950	$23,990
TBN Research Technician III	Operator	12	40%	34,500	$13,800	$3,588	$17,388
				SUBTOTALS	$146,973	$38,213	$185,185

CONSULTANT COSTS	$0
EQUIPMENT *(Itemize)*	$0
SUPPLIES *(Itemize by category)* Membranes for Cross flow microfiltration and ultrafiltration studies (bench and pilot scale) 20,000 Chromatography Resins for pilot scale runs, 45,000 Disposables, 45,000 (purchased in year 1) Facility Change Over/Cleaning Supplies, 17,700	$82,700
TRAVEL	0
PATIENT CARE COST — INPATIENT	
PATIENT CARE COST — OUTPATIENT	
ALTERATIONS AND RENOVATIONS (Itemize by category)	
OTHER EXPENSES (Itemize by category)	$0
SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD	$267,885
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*	**$267,885**
SBIR/STTR Only: FEE REQUESTED	

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

cGMP Production (UNL)

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		0	0	0	185,185	190,741
CONSULTANT COSTS		0	0	0	0	
EQUIPMENT		0	0	0	0	0
SUPPLIES		45,000	34,500	0	82,700	35,000
TRAVEL		0	0	0	0	0
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		0	0	0	0	0
SUBTOTAL DIRECT COSTS		45,000	34,500	0	267,885	225,741
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	F&A					
TOTAL DIRECT COSTS		45,000	34,500	0	267,885	225,741
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$573,126
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Proppposed Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

PERSONNEL

Scott Johnson, M.S., cGMP Production Coordinator

Mr. Johnson will spend 50% of his time on the project managing the cGMP production of Factor IX. Operation and management of a cGMP facility requires a significant amount of oversight just to maintain the facility and to continually upgrade GMPs. It is the c in cGMP (current) that makes operation of a facility so labor-intensive. Managing such a facility includes interacting with Ms. Barthuli, Quality Assurance Coordinator of the Biological Process Development Facility (BPDF) and the Quality Control Laboratory, which performs all of the testing of environmental and water samples. Mr. Johnson will also have primary responsibility for writing the Production Batch Records. Mr. Johnson will be responsible for scheduling the cGMP runs, staffing the production runs, first review of the completed Production Batch Records, and being on the floor during the runs.

cGMP Production, Budget Justification, continued

Five Research Technologists I
The research technologists (operators) will be responsible for running the cGMP production process. To meet cGMPs, two operators must be on the floor for all critical operations when verification is required. These technologists are also responsible for cleaning and maintaining the pilot plants. In the budget are listed Research Technologists for FPP and PPP. FPP stands for Fermentation Pilot Plant, which is where the initial recovery will be accomplished. The recovered Factor IX is then sterile-filtered into the Purification Pilot Plant (PPP) where the purification, polishing, and final formulation are completed.

Research Technician III. This individual's primary responsibility is daily cleaning of the pilot plants and sampling of water and the environment.

SUPPLIES COSTS

Membranes: Membranes will be required for buffer exchange, concentration, and virus removal. Membranes maybe required for recovery, depending on the results from process development studies. Modules costs can vary from $2,000 to $10,000 per module depending on flux rate and type.

Chromatography Resins: New chromatography resins (5 to 10 L) are necessary for cGMP production. It is our intent to reuse the resins for the second run based on studies during purification development. If not, resources will be needed to use fresh resins.

Disposables: This budget is for disposables that are used in the cGMP pilot plants, such as dead end filters, plastic tank bag liners, final product storage containers, etc. This category also covers all of the testing (water and environment) supplies for maintaining the GMPs of the facility before, during, and after Factor IX processing. The pilot plants are monitored on a daily and weekly basis. In order to balance the budget in accordance with the requirements of the RFA, disposable production supplies that would normally be purchased in years 4 and 5 will be purchased in years 1 and 2.

Facility Change Over and Cleaning Supplies: The cGMP pilot plants are cleaned on a daily and weekly basis. Prior to the cGMP production, all elastomers are replaced. This process takes approximately 10 to 14 working days to complete and requires that all elastomers be changed, such as o-rings and gaskets, including braided sanitary tubing. The average cost just for elastomers is approximately $10,000.

Quality Assurance (UNL)

Principal Investigator/Program Director (Last, first, middle): Velander, William H.

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY					FROM 1/1/2005		THROUGH 12/31/2005
Personnel *(Applicant organization only)*			%		DOLLAR AMOUNT REQUESTED *(omit cents)*		
NAME	ROLE ON PROJECT	TYPE APPT. (months)	EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Ardis Barthuli	QA coord	12	40%	70,000	$28,000	$7,280	$35,280
TBN QA Doc Specialist I	Scientist	12	80%	48,000	$38,400	$9,984	$48,384
TBN QA Doc Specialist I	Scientist	12	80%	48,000	$38,400	$9,984	$48,384
				SUBTOTALS	$104,800	$27,248	$132,048

Category	Amount
CONSULTANT COSTS	$0
EQUIPMENT *(Itemize)*	$0
SUPPLIES *(Itemize by category)* Equipment Calibration, 12,000 Document Processing costs, 8,000	20,000
TRAVEL ACS or Pittcon Meeting--2 people	4000
PATIENT CARE COST — INPATIENT	
PATIENT CARE COST — OUTPATIENT	
ALTERATIONS AND RENOVATIONS (Itemize by category)	
OTHER EXPENSES (Itemize by category)	0
SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD	$156,048
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*	**$156,048**

SBIR/STTR Only: FEE REQUESTED

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

Quality Assurance (UNL)

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		132,048	136,009	140,090	144,292	148,621
CONSULTANT COSTS		0	0	0	0	
EQUIPMENT		0	0	0	0	0
SUPPLIES		20,000	20,600	21,218	21,855	22,510
TRAVEL		4,000	4,120	4,244	4,371	4,502
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		0	0	0	0	0
SUBTOTAL DIRECT COSTS		156,048	160,729	165,551	170,518	175,633
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	F&A					
TOTAL DIRECT COSTS		156,048	160,729	165,551	170,518	175,633
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$828,480
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Proppposed Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

PERSONNEL

Ardis Barthuli, Quality Assurance Coordinator

Ms. Barthuli is responsible for the entire Quality Assurance program of the UNL Biological Process Development Facility and will dedicate 40% of her time to this project. Ms. Barthuli will be responsible for overseeing the QA document specialists that will be writing and editing all of the Standard Operating Procedures (SOPs) that will be generated from Analytical Methods Development and the Production Batch Records. Because this project will be utilizing the cGMP pilot plants and the Quality Control Laboratory, Ms. Barthuli's time will also be required as regular oversight of all QA issues related to cGMP activities in the pilot plants and Quality Control Laboratory.

UNL Quality Assurance Program

PERSONNEL
Ardis Barthuli, Quality Assurance Coordinator
Ms. Barthuli is responsible for the entire Quality Assurance program of the UNL Biological Process Development Facility and will dedicate 40% of her time to this project. Ms. Barthuli will be responsible for overseeing the QA document specialists that will be writing and editing all of the Standard Operating Procedures (SOPs) that will be generated from Analytical Methods Development and the Production Batch Records. Because this project will be utilizing the cGMP pilot plants and the Quality Control Laboratory, Ms. Barthuli's time will also be required as regular oversight of all QA issues related to cGMP activities in the pilot plants and Quality Control Laboratory.

TBN, Quality Assurance Document Specialists
This project will require two QA Document Specialists with 80% effort for the entire project to assist in writing, editing, and tracking all cGMP documents and technology transfer documents related to this project.

SUPPLIES COSTS
Equipment Calibration: This is to cover the cost of supplies, outside contractors, and NIST traceable instruments to calibrate equipment used for this grant.

Document Processing: This is to cover the cost of binders, paper, printing, copying, and document equipment supplies (scanner, copier, fax machine, and shredder).

TRAVEL
This is to cover the cost of two QA professionals to attend a national meeting.

Quality Control (UNL)

Principal Investigator/Program Director (Last, first, middle): Velander, William H.

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY					FROM 1/1/2006		THROUGH 12/31/2006
Personnel *(Applicant organization only)*					DOLLAR AMOUNT REQUESTED *(omit cents)*		
NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
S. Todd Swanson	co-PI	12	10%	71,585	$7,159	$1,861	$9,020
TBN Research Tech I	Scientist	12	90%	45,000	$40,500	$10,530	$51,030
TBN Research Tech I	Scientist	12	50%	45,000	$22,500	$5,850	$28,350
				SUBTOTALS	$70,159	$18,241	$88,400

Category	Amount
CONSULTANT COSTS	$0
EQUIPMENT *(Itemize)*	$0
SUPPLIES *(Itemize by category)* Western and ELISA antibodies and reagents, 25091 HPLC Columns, 27,909 HPLC Solvents, 23,906 HPLC Supplies and Chemicals, 8,010 General Laboratory Chemicals, 8,395 General Laboratory Supplies, 8,937 Electrophoresis supplies, 3,000	105,248
TRAVEL ACS or Pittcon Meeting	2000
PATIENT CARE COST — INPATIENT	
PATIENT CARE COST — OUTPATIENT	
ALTERATIONS AND RENOVATIONS (Itemize by category)	
OTHER EXPENSES (Itemize by category) Mass Spectrometry Contract Fees: $3,000; Cost of HPLC maintainence contracts: $12,000	15,000
SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD	$210,648
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*	**$210,648**

SBIR/STTR Only: FEE REQUESTED

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

Quality Control (UNL)

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		88,400	198,483	150,299	154,808	159,452
CONSULTANT COSTS		0	0	0	0	
EQUIPMENT		0	0	0	0	0
SUPPLIES		105,248	57,342	75,700	33,630	21,868
TRAVEL		2,000	2,060	2,122	2,185	2,251
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		15,000	17,500	20,500	21,115	21,748
SUBTOTAL DIRECT COSTS		210,648	275,385	248,621	211,738	205,319
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	F&A					
TOTAL DIRECT COSTS		210,648	275,385	248,621	211,738	205,319
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$1,151,711
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Proppsoed Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

PERSONNEL

S. Todd Swanson, Ph.D., Key Person

Dr. Swanson will devote 20% of his effort to this project as Co-Investigator and Key Person. He will have the overall responsibility for supervision of all aspects of Quality Control and will ensure that the needs of the project for Quality Control services are met. He will design and supervise the stability testing studies of bulk product and final formulations in conjunction with the Administrative, Quality Assurance, Production, and Formulation groups. He will supervise the research technologists and the research technicians, who will provide technical support. He will review all Quality Control data and approve or reject their release from the laboratory. He will also be responsible for facilitating communication of data and results from the Quality Control Laboratory among all interested parties. He will be responsible for resolving all issues of assay performance and reliability. Dr. Swanson has industrial experience in Quality Control and expertise in high-performance liquid chromatography of proteins. He will also be involved in review of technology transfer documents.

UNL Quality Control Laboratory, Budget Justification, Continued

TBN Research Technologist I #1, Quality Control Laboratory Factor IX team leader
This individual will devote 90% of his/her effort to this project for all five years. She/he will act as the main repository of knowledge for Factor IX-related testing for the Quality Control Laboratory. In the first two years of the project, this person will focus on three major tasks: 1) technical transfer of assays created by the FIX Analytical Methods Group into the Quality Control Laboratory; 2) qualification of the transferred methods i.e., ensuring the transferred method functions as intended in the Quality Control Laboratory by actual testing; and 3) training of other Quality Control Laboratory staff to perform these methods. These tasks will require the following: 1) authoring, editing, and reviewing of standard operating procedures; 2) specifying, ordering, installation, and testing of specific analytical systems; 3) implementing new testing procedures; and 4) training of other Quality Control staff to use these procedures. This person will be skilled with all instrumentation in the laboratory and capable of troubleshooting problems. In Years 3-5, he/she will also provide routine technical support and QC services to process development and manufacturing elements of this project. She/he will provide a first level of supervision for the Research Technicians (see below). She/he will also perform record keeping duties necessary for maintaining a laboratory in compliance with current Good Laboratory Practice.

TBN Research Technologist I #2
This individual will devote 50% of his/her effort to this project in the first year and 90% of his/her effort in the second year with his/her participation ending after Year 2. During the time of participation, she/he will assist the Quality Control Laboratory FIX team leader (Research Technologist I #1 above) with the technical transfer, qualifications, and training necessary to implement the new analytical procedures in the Quality Control Laboratory. She/he will assist the team leader with procedure testing and troubleshooting. The 40% increase in FTE on Year 2 will be used for training the Research Technicians (see below) who start in Year 2.

TBN Research Technician III – 2 FTEs
These individuals will devote 90% of their effort to this project. They will provide routine technical support to the process development and production efforts. This will be accomplished by using the transferred assays developed by the FIX analytical methods group. They will also perform record keeping duties necessary for maintaining a laboratory in compliance with current Good Laboratory Practice.

Fringe benefits for permanent staff are calculated as 26% of salary.

SUPPLIES COSTS
($105,248 in Year 1; $57,342 in Year 2; $75,700 in year 3; $33,630 in year 4; and $21,868 in year 5)

The following figures are derived through cost-accounting, i.e., using actual purchase orders written over the course of one year to purchase supplies for a similar project. In order to balance the budget in accordance with the requirements of the RFA, some supplies that would normally be to be purchased in years 4 and 5 will be purchased in years 1 and 3.The monies requested here will purchase the following items:

HPLC: We expect that a minimum of 1,843 samples will be analyzed by a quantitative HPLC method per year during process development and production. This estimate is based on the use of more than one method being applied to the same sample, e.g., one sample evaluated by both size exclusion and reverse phase methods. This estimate also includes triplicate analysis of any sample that requires quantitation with an error estimate. Approximately 25% of these samples will be “dirty” (early stage purification from whole milk forward), and this type of sample substantially shortens column life. Note that HPLC costs are fixed except for adjustments for inflation in Year 2. Year 1 expenditures are less because routine technical support for process development and production does not begin until Year two. Year 1 costs cover the process of technical transfer, qualification, and training for analytical methods.

HPLC columns This category includes the following: size exclusion columns with guard columns; reverse phase columns (C4, C8, C18) with guard columns; cation exchange columns with guard columns; anion exchange columns with guard columns; and hydrophobic interaction chromatography columns. Also included in this category are coated and uncoated capillaries for the various modes of capillary electrophoresis.

HPLC solvents High purity water (low UV contaminate low carbon load); Optima grade Acetonitrile; Optima grade Methanol; and HPLC grade Isopropanol.

HPLC supplies and chemicals ($8,010): This category includes the following: Autosampler vials; tubing, fittings, inline filters; sample & solvent filtration, solid phase extraction for sample clean up, HPLC grade mobile phase chemicals, e.g., formic acid & trifluoroacetic acid for reverse phase chromatography, various salts for ion exchange and hydrophobic interaction chromatography. Also included in this category are the fluorescent labeling reagents for the LIF mode of detection for capillary electrophoresis.

Limulus Amoebocyte Lysate (LAL) Assay for Pyrogens ($5,000, expenditure starts in Year 2): Costs include the following: Limulus amoebocyte lysate, Endotoxin free buffers, reference standard Endotoxin, and the shipping of these materials. This testing is critical during process development and production to ensure a very low to zero Endotoxin burden in the final product. This test will be used to monitor Endotoxin burden from the beginning of the process to the final product.

Antibodies and Reagents for Western Blots and ELISA assays Costs include the following: Commercially available primary and secondary antibodies; cost of subcontracting for creation of non-commercially available primary antibodies; kits, reagents, and supplies necessary for detection of antibody binding; disposables associated with these techniques (e.g., 96 well plates and PVDF membranes); and the shipping of these materials. These techniques will be used to support of process development and production.

Laboratory Chemicals ($8,395): This category includes the following: reagents for total protein assays, high purity buffers, salts, detergents, amino acids, non-HPLC solvents, and other general chemicals. Additionally, this includes reagents, media, and other chemicals needed for microbial testing in support of late stage process development and production.

General Laboratory Supplies ($8,937): This category includes disposables (glass tubes, plastic ware, sterile filter apparatuses, 96 well microtiter plates, pipettes, pipettor tips, centrifuge ultrafiltration tubes, syringes, etc.), other consumables, and other general laboratory supplies. This includes the costs of labels, log books, notebooks, and other materials necessary to maintain the cGLP status of the Quality Control Laboratory. Included as well are the costs of spin desalting columns necessary for buffer exchange. Additionally, this includes disposables and other consumables needed for microbial testing in support of late stage process development and production.

Electrophoresis Supplies ($3,000): To maintain consistent results with electrophoresis, we purchase pre-cast gels and pre-made buffers and stains. We do this because the manufacturers control variation between lots. It is critical to have gels with consistent analytical performance over time for the stability studies. We have standardized on Novex (Invitrogen) as an electrophoresis supplier. This category includes the following materials: Pre-cast SDS-PAGE gels, pre-cast IEF gels, IPG strips, electrolytes, electrophoretic standards, pre-made buffers, pre-made stains, and materials for drying and archiving.

TRAVEL
($2,000)
One trip per year for one person (the co-investigator or the scientists) is requested to attend a national scientific meeting (ACS or Pittcon: Registration: $300; Hotel: $1,000 {5 days}, Airfare: $600, ground transportation: $100). The purposes of this trip will be to present data acquired under this grant, confer with colleagues in the same field, and to learn about new technologies that may apply to future needs of the project.

OTHER
($15,000 in Year 1, $17,500 in Year 2, $20,500 subsequent years)

Analytical Services: All of these services require equipment and expertise that we do not possess. All of these services cannot be provided by us but are critical characterization and analysis that must be done in order to advance Factor IX to clinical trials. It is more cost- and time-effective to contract for these services than it is to acquire the equipment and expertise ourselves.

Mass Spectrometry Services ($3,000 Years 1 & 2, $5,000 subsequent years): This covers the annual cost of analysis and characterization of purified Factor IX by various forms of mass spectrometry. Mass spectrometry is a key analytical technique that is critical for characterization of factor IX at all stages of development, production, and formulation.

Protein Sequencing Services ($1,000 Year 1, $1,500 Year 2, and $2,500 subsequent years): This covers the annual cost of analysis and characterization of purified Factor IX by amino-terminal amino acid sequencing. Amino-terminal amino acid sequencing is a key analytical technique that is critical for characterization of Factor IX at all stages of development, production, and formulation.

Amino Acid Analysis Services ($1,000): This covers the annual cost of analysis of purified Factor IX by amino acid analysis. Amino acid analysis is the most reliable, most direct method to quantitate a protein. Knowledge of exact protein concentrations is critical for characterization of Factor IX in the final stages of development, production, and formulation.

Cost of HPLC maintenance and qualification contracts (prorated to 30%) $12,000: This service covers the annual preventive maintenance by the manufacturer's field service engineer and guarantees the repair of any system that fails. This is provided at a cost that is slightly more than the cost of the preventive maintenance kits that can be purchased from the manufacturer. These costs also include the validation of the HPLC systems, the Client/Server database, and the data acquisition and instrument control software. This project will use HPLC as a primary analytical method in multiple types of assays.

UNL Project Management

Principal Investigator/Program Director (Last, first, middle): Velander, William H.

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY

FROM 1/1/2005 THROUGH 12/31/2005

Personnel *(Applicant organization only)* — DOLLAR AMOUNT REQUESTED *(omit cents)*

NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
William Velander	Principal Investigator	12	25%	146,000	$36,500	$9,490	$45,990
Michael Meagher	co-PI	12	20%	132,500	$26,500	$6,890	$33,390
TBN, Program Coordinator	Admin.	12	90%	51,000	$45,900	$11,934	$57,834
Kevin McCulloch	Maintenance	12	40%	46,000	$18,400	$4,784	$23,184
William Heidal	Info Tech	12	40%	59,000	$23,600	$6,136	$29,736
				SUBTOTALS	$150,900	$39,234	$190,134

Category	Amount
CONSULTANT COSTS	$0
EQUIPMENT *(Itemize)*	$0
SUPPLIES *(Itemize by category)* CyberLab Maintenance Computer Supplies Maintenance Supplies	$24,000
TRAVEL	12000
PATIENT CARE COST — INPATIENT	
PATIENT CARE COST — OUTPATIENT	
ALTERATIONS AND RENOVATIONS (Itemize by category)	
OTHER EXPENSES (Itemize by category)	$0
SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD	$226,134
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*	**$226,134**

SBIR/STTR Only: FEE REQUESTED

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

UNL Project Management

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		190,134	195,838	201,713	207,765	213,997
CONSULTANT COSTS		0	0	0	0	
EQUIPMENT		0	0	0	0	0
SUPPLIES		24,000	24,720	25,462	26,225	27,012
TRAVEL		12,000	12,360	12,731	13,113	13,506
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		0	0	0	0	0
SUBTOTAL DIRECT COSTS		226,134	232,918	239,906	247,103	254,516
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	F&A					
TOTAL DIRECT COSTS		226,134	232,918	239,906	247,103	254,516
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$1,200,576
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Propposed Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

PERSONNEL

William Velander, Ph.D., Principal Investigator

Dr. Velander is the Principal Investigator and will dedicate 25% of his time to the project. As Principal Investigator, Dr. Velander will be responsible for the development and supervision of the entire program including, but not limited to, providing technical oversight, designing experiments, interpreting data, and attending meetings. Dr. Velander will also serve as the project's point of contact.

UNL Project Management, Budget Justification, continued

Michael Meagher, Ph.D., Lead Scientist and Co-Investigator
Dr. Meagher's expertise is in process development and current Good Manufacturing Practice production of recombinant proteins. He is the Director of the University of Nebraska-Lincoln Biological Process Development Facility (BPDF). Dr. Meagher will oversee all aspects of process development and cGMP manufacturing of Factor IX. Dr. Meagher will have immediate responsibility for ensuring that Factor IX produced in the BPDF cGMP Facility meets final product specifications. Dr. Meagher will dedicate 20% of his time to this project.

TBN: Program Coordinator
This individual will be dedicated 90% of his/her time to the coordinating of non-scientific activities that will be ongoing primarily at the University of Nebraska-Lincoln, but also will interact with Lead Investigators at the University North Carolina and at Colorado State University. This person will oversee the administrative activities of the grant, in particular, all aspects of report submission, which includes reviewing all written reports, making sure reports meet NIH's reporting requirements, and coordinating all reporting activity with each of the Lead Investigators. This person will be responsible for scheduling weekly conference calls, participating in these weekly calls, and will be the lead person for making sure all action items from these meetings are addressed during that week. Other duties include scheduling, coordinating, and participating in quarterly face-to-face meetings, and monitoring progress of action items from these meetings.

Kevin McCulloch, Maintenance
Mr. McCulloch is responsible for maintaining equipment in the University of Nebraska-Lincoln Biological Process Development Facility (BPDF). This includes the Purification Development Laboratory (three BioCad chromatography systems, one pilot-scale chromatography system, and one membrane system), Analytical Development Laboratory, Quality Control Laboratory, and Media Prep Room (two autoclaves and a dishwasher). Because the BPDF operates under GLP, all of this equipment (and others not specifically listed) require routine maintenance and immediate repair. This project is programmed to use nearly one-third of the available resources at the BPDF. This individual is critical to keeping the facility running.

William Heidal, Information Technology
The UNL component of this project will be electronic with respect to data archiving and transfer of electronic information, and all critical equipment is interfaced with computers. The BPDF has more than 40 personal computers (equipment and personal), five servers, and a two-terabyte Storage Area Network (SAN) available to this project. Mr. Heidal has the responsible of maintaining and upgrading computer systems in the BPDF, of which a significant number will be serving this project.

SUPPLIES COSTS
CyberLab: This category covers the license fees for five project personnel to have simultaneous access to Cyber lab, the BPDF electronic notebook system. Additional costs include repair parts (hard drives, monitors, etc.) and software licensing fees for such programs as Microsoft Project, Adobe Acrobat, etc.

Maintenance Supplies: This category covers the cost of general supply costs of maintenance and calibration of equipment.

TRAVEL
This category covers the cost of travel to NIH and one professional meeting for Dr. Velander, Dr. Meagher, and the program coordinator.

CSU--Formulation Studies

Principal Investigator/Program Director (Last, first, middle): Velander, William H.

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY					From 12/1/2005		Through 12/31/2006
Personnel (Applicant organization only)					**Dollar Amount Requested (omit cents)**		
NAME	ROLE ON PROJECT	TYPE APPT. (Months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Mark Manning	co-PI	12	25%	103,000	$25,750	$6,695	$32,445
William S. Dernell	co-PI	12	10%	92,004	$9,200	$2,392	$11,592
Patrick Miller	Post Doc	12	100%	48,000	$48,000	$12,480	$60,480
				SUBTOTALS	$82,950	$21,567	$104,517

CONSULTANT COSTS	$0
EQUIPMENT (Itemized) Agilent 8453 UV Spectrophotometer, 22,000 DynaPro DLS, 26,000	$48,000
SUPPLIES (Itemize by category) Glassware and Chemicals	$7,200
TRAVEL Travel to meet with collaborators	$3,000
PATIENT CARE COST — INPATIENT	
PATIENT CARE COST — OUTPATIENT	$0
ALTERATIONS AND RENOVATIONS (Itemize by category)	$0
OTHER EXPENSES (Itemize by category)	$0 $600
SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD	$163,317
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS @ 45.0%	$51,893
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page)	**$215,210**
SBIR/STTR Only: FIXED FEE REQUESTED	

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

CSU Formulation Studies

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		104,518	0	130,955	0	0
CONSULTANT COSTS		0	0	0	0	
EQUIPMENT		48,000	0	0	0	0
SUPPLIES		7,200	0	11,160	0	0
TRAVEL		3,000	0	3,090	0	0
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		600	0	33,000	0	0
SUBTOTAL DIRECT COSTS		163,318	0	178,205	0	0
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	F&A	51,893	0	80,192	0	0
TOTAL DIRECT COSTS		215,211	0	258,397	0	0
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$473,608
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Propposed Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Please see the following page:

Colorado State University – Formulation Studies

PERSONNEL
Mark Manning, Ph.D., Lead Investigator
Dr. Manning will oversee the transgenic Factor IX formulation for intravenous and oral administration. He will direct protein structure and function studies that are used to evaluate the formulations. He will dedicate 25% of his time during the formulation experiments, and a salary is requested for his efforts.

William Dernell, M.S., D.V.M., Key Person
Dr. Dernell will oversee the evaluation of the IV and oral formulations in normal mouse and normal dog models. He will dedicate 10% of his time during the formulation experiments, and a salary is requested for his efforts.

Patrick Miller, Ph.D., and Alexandra De Lille, Ph.D., Post-doctoral Fellows
The post-doctoral fellows will be responsible for conducting the protein formulation experiments, analyzing the properties of the transgenic Factor IX formulations, and conducting the animal model treatments, sample collection, and sample analysis. Dr. Miller will be spending 100% of his time on this project, and Dr. De Lille will be spending 17% of her time on this project. Salaries are requested.

Laura Chubb, Technician
The technician will dedicate 25% of her time to this project and assist with the animal model experiments.

EQUIPMENT
Agilent 8453 UV Spectrophotometer: GLP diode array spectrophotometer needed for protein concentration and enzymatic assays.

DynaPro DLS Instrument: For dynamic light-scattering experiments to measure the hydrodynamic radius of transgenic Factor IX molecules; used to determine if high molecular weight aggregates are present.

SUPPLIES COSTS
General Laboratory Supplies: This is to cover the costs of reagents such as chemicals, dry goods, electrophoresis gels and reagents, glassware, animal medical supplies (needles, syringes, plasma and serum collection tubes, etc.).

OTHER
Animal Costs: Procurement ($14.20/mouse; $400/dog), shipping ($54 for mice; $800 for dogs), housing ($0.66/day/cage for mice; $3.38/day/dog), and histology ($20/mouse; $50/dog) for mice and dogs. It is estimated that at least 60 mice and 12 dogs will be needed for these studies.

Drug Analysis: Laboratory evaluation for dog serum and urine samples are $45/sample; Factor IX analysis is estimated at $25/sample.

Publication Costs: Publication and dissemination of data.

UNC Chapel Hill--Mouse Model Experiments

Principal Investigator/Program Director (Last, first, middle): Velander, William

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY

From 12/1/2005 Through 12/31/2006

Personnel (Applicant organization only)					Dollar Amount Requested (omit cents)		
NAME	ROLE ON PROJECT	TYPE APPT. (Months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Paul Monahan	P.I	12	10%	103,703	$10,370	$2,385	$12,755
Tai-ping Zhang	post doc	12	50%	36,108	$18,054	$4,152	$22,206
Nathan LaBorde	technician	12	20%	22,660	$4,532	$1,042	$5,574
				SUBTOTALS	$32,956	$7,579	$40,535

Item	Amount
CONSULTANT COSTS	$0
EQUIPMENT (Itemized)	$0
SUPPLIES (Itemize by category) Mice, Anesthesia/Vet supplies--Isofluorane Surgifoam Assays: antibodies: anti-human F.IX, anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA additional antibody supplies (colorigenic reagent), human plasma standards mouse immunoglobin standards, plasma-derived human factor IX control Coagulation factor IX: Commercial, Recombinant (Benefix) Commercial, plasma-derived, monoclonal antibody purified (Mononine) Coagulometer supplies: Cuvettes: 3 boxes Steel balls: 1 APTT reagent: 2 Buffers (2) Unicalibrator: 3 Thermal paper, CaCl, Finntipps: Plastics, Disposables	$10,172
TRAVEL Travel to meet with collaborators	$800
PATIENT CARE COST — INPATIENT	
PATIENT CARE COST — OUTPATIENT	$0
ALTERATIONS AND RENOVATIONS (Itemize by category)	$0
OTHER EXPENSES (Itemize by category)	$0 $0
SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD	$51,507
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS @ 46.0%	$23,693
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page)	**$75,201**
SBIR/STTR Only: FIXED FEE REQUESTED	

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

UNC Chapel Hill--Mouse Model Experiments

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		40,536	0	44,046	45,675	0
CONSULTANT COSTS		0	0	0	0	
EQUIPMENT		0	0	0	0	0
SUPPLIES		10,171	0	10,244	10,321	0
TRAVEL		800	0	824	849	0
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		0	0	0	0	0
SUBTOTAL DIRECT COSTS		51,507	0	55,114	56,845	0
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	F&A	23,693	0	25,352	26,149	0
TOTAL DIRECT COSTS		75,200	0	80,466	82,994	0
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$238,660
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Proppposed Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Please see the following page:

Budget Justification: Hemophilia B Mouse Model Experiments (UNC-Chapel Hill)

PERSONNEL

Paul Monahan, M.D., Lead Investigator
Dr. Monahan will spend 10% of his time directly conducting experiments with transgenic rhFIX in the murine hemophilia B model, and requests a salary. Dr. Monahan will be responsible for supervising a post-doctoral fellow and a technician, overseeing the animal experiments to ensure compliance, designing the experiments, interpreting the results, and communicating the results to other Lead Investigators and project team members. A 3%/year increase is included.

Tai-ping Zhang, MD., **Post-doctoral Fellow**
Dr. Zhang will conduct the experiments for intravenous and oral dosage in the hemophilia B mouse model, and a salary is requested. Dr. Zhang is a gastrointestinal surgeon who will be continuing a research sabbatical from the Surgery faculty of Peking Union Medical School. Dr. Zhang will spend 50% of his time on this project, and will be responsible for conducting experiments, animal procedures and post-procedural care, assisting with experimental design, assisting with interpretation of the results, and preparation of reports. A 3%/year increase is included.

Nathan LaBorde, Research Technician
Mr. LaBorde will assist in maintenance of the hemophilia B mouse colony that is used in this project, and a salary is requested. He will spend 20% of his time on this project, and will be responsible maintaining hemostatically normal and hemostatically deficient mouse colonies, screening for genotype and for the bleeding phenotypes, and for feeding and care of the animals. A 3%/year increase is included.

SUPPLIES COSTS

Mouse cage costs: Covers the animal facility per diem costs ($2.98/wk for mice in a hot washed cage). A 4%/year increase is included for subsequent years.

Anesthesia and Vet Supplies: Covers the costs of Isoflurane, Surgifoam, and the maintenance of inhalation anesthesia equipment and operative space.

Immunoassays: Covers costs for anti-human FIX antibodies, antibodies to mouse immunoglobulins (to determine if inhibiting antibodies are present), colorigenic reagents, human FIX standards, mouse immunoglobulin standards.

Coagulation assays: Covers costs for BeneFIX ($1600), Mononine ($1600), cuvettes ($600), steel balls ($280), aPTT reagent, buffers (Owren's buffer, calcium chloride), coagulation meter supplies (thermal paper, pipet tips).

General lab supplies: Covers costs of routine disposables (pipets, pipet tips, microcentrifuge tubes, etc.).

TRAVEL
$800/year is requested for travel to meet with Lead Investigators in Lincoln, NE.

UNC Chapel Hill--Dog Trials

Principal Investigator/Program Director (Last, first, middle): Velander, William H.

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY					From 12/1/2007		Through 12/31/2008
Personnel (Applicant organization only)					Dollar Amount Requested (omit cents)		
NAME	ROLE ON PROJECT	TYPE APPT. (Months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Timothy Nichols	P.I	12	15%	170,625	$25,594	$5,349	$30,943
Elizabeth Merricks	Res. Analyst	12	90%	54,434	$48,991	$12,409	$61,400
							$0
				SUBTOTALS	$74,584	$17,758	$92,342

CONSULTANT COSTS	$0
EQUIPMENT (Itemized)	$0
SUPPLIES (Itemize by category) 4 hemophilia B dogs at $5.97/day x 365 days + classification charge /dog, 9,116 Processing, preparing blood samples, anticoagulants, reagents for assaying FIX, 15,000 Serum chemistries, shipping, plastics, etc., 9,000	$33,116
TRAVEL Travel to meet with collaborators	$0
PATIENT CARE COST — INPATIENT	
PATIENT CARE COST — OUTPATIENT	$0
ALTERATIONS AND RENOVATIONS (Itemize by category)	$0
OTHER EXPENSES (Itemize by category)	$0 $0
SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD	$125,458
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS @ 46.0%	$57,711
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page)	**$183,169**
SBIR/STTR Only: FIXED FEE REQUESTED	

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

UNC Chapel Hill--Mouse Model Experiments

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		0	0	92,342	0	95,113
CONSULTANT COSTS		0	0	0	0	
EQUIPMENT		0	0	0	0	0
SUPPLIES		0	0	33,116	0	68,881
TRAVEL		0	0	0	0	0
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		0	0	0	0	0
SUBTOTAL DIRECT COSTS		0	0	125,458	0	163,994
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	F&A	0	0	57,711	0	75,437
TOTAL DIRECT COSTS		0	0	183,169	0	239,431
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$422,600
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Proppoosed Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Please see the following page:

Budget Justification: Hemophilia B Canine Model Experiments (UNC-Chapel Hill)

PERSONNEL

Timothy C. Nichols, M.D., Lead Investigator
Professor of Medicine and Pathology and Laboratory Medicine, is the Lead Investigator. He will plan and supervise all of the collaborative research activities and will spend 15% of his time conducting the experiments with transgenic rhFIX in the canine hemophilia B model, and requests salary support. Dr. Nichols will be responsible for supervising Ms. Elizabeth Merrricks, overseeing the animal experiments to ensure compliance, designing the experiments, interpreting the results, and communicating the results to other Lead Investigators and project team members. He is the laboratory director of the Francis Owen Blood Research Laboratory (FOBRL). He has worked with the bleeder animals at the FOBRL since 1982. He maintains an office and laboratory at the FOBRL. A 3%/year increase is included.

Elizabeth Merricks, B.S., Research Analyst
Dr. Merricks will be responsible for the analysis of all samples obtained from the dogs for classification and before and after treatment of spontaneous bleeding episodes, dosing and administration of vectors and other test reagents and collaborative protocols. Her responsibilities will include performing related clotting assays, electrophoretic procedures, ELISA quantitation of F.IX antigen, SDS-PAGE analysis of F.IX, and analysis of F.IX activity and Bethesda Inhibitors as needed.. She will maintain the scientific records of these results. She will spend 90% of their time on this project, and will be responsible for conducting experiments, assisting with experimental design, assisting with interpretation of the results, and preparation of reports. A 3%/year increase is included.

SUPPLIES COSTS
Animal costs: Covers the animal facility per diem costs ($5.97/day plus classification charge/dog). A 4%/year increase is included for subsequent years.

Coagulation assays: Covers costs for processing and preparing blood samples, aPTT reagent, buffers, coagulation meter supplies, plastic ware, coagulation reagents, dry ice, liquid N2, catheters, histological processing of biopsies, CBC analyses, serum chemistry analyses for monitoring liver enzymes, serum electrolytes, and for evidence of a procoagulant effect.

LifeSci Partners

Principal Investigator/Program Director (Last, first, middle): Velander, William

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY					FROM 1/1/2005		THROUGH 12/31/2005
Personnel (Applicant organization only)					Dollar Amount Requested (omit cents)		
NAME	ROLE ON PROJECT	TYPE APPT. (Months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Stephen Abramson	co-P.I	12	35%	150,000	$52,500	$13,650	$66,150
				SUBTOTALS	$52,500	$13,650	$66,150

CONSULTANT COSTS	$0
EQUIPMENT (Itemized)	$0
SUPPLIES	
TRAVEL Tavel to project sites (10 trips/yr at 2,000/trip)	$20,000
PATIENT CARE COST — INPATIENT	
PATIENT CARE COST — OUTPATIENT	$0
ALTERATIONS AND RENOVATIONS (Itemize by category)	$0
OTHER EXPENSES (Itemize by category)	$0 $0
SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD	$86,150
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS @ 44.0%	$37,906
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page)	**$124,056**
SBIR/STTR Only: FIXED FEE REQUESTED	

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

LifeSci Partners

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		66,150	68,135	70,179	72,284	74,452
CONSULTANT COSTS		0	0	0	0	
EQUIPMENT		0	0	0	0	0
SUPPLIES		0	0	0	0	0
TRAVEL		20,000	20,600	21,218	21,855	22,510
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		0	0	0	0	0
SUBTOTAL DIRECT COSTS		86,150	88,735	91,397	94,138	96,963
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT	86,150	88,735	91,397	94,138	96,963
	F&A	37,906	39,043	40,214	41,421	42,664
TOTAL DIRECT COSTS		124,056	127,778	131,611	135,559	139,626
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$658,630
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Proppposed Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

PERSONNEL

Stephen Abramson, Ph.D., Lead Investigator
Dr. Abramson will dedicate 35% of his effort to this project. Dr. Abramson has a tremendous amount of experience in the biopharmaceutical industry and was instrumental in bringing plasma-derived Factor IX to commercial production. Dr. Abramson will assist Dr. Velander in all aspects of the project, especially issues related to bring transgenic human Factor IX to clinical trials.

TRAVEL COSTS

Dr. Abramson will be traveling to Nebraska, North Carolina, Colorado, and NIH during the project. Dr. Abramson is located in California and will be making 10 trips per year to the different sites as needed.

Pro Genetics, LLC

Principal Investigator/Program Director (Last, first, middle): Velander, William

DETAILED BUDGET FOR INITIAL BUDGET DIRECT COST ONLY					From 1/1/2005		Through 12/31/2005
Personnel (Applicant organization only)			%		Dollar Amount Requested (omit cents)		
NAME	ROLE ON PROJECT	TYPE APPT. (Months)	EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Julian Cooper	co-P.I	12	0%	0	$0	$0	$0
				SUBTOTALS	$0	$0	$0

Category	Amount
CONSULTANT COSTS	$0
EQUIPMENT (Itemized)	$0
SUPPLIES (Cost to Maintain and Supply Transgenic Milk from 5 Female Lactating Pigs)	$62,000
TRAVEL	$0
PATIENT CARE COST — INPATIENT	
PATIENT CARE COST — OUTPATIENT	$0
ALTERATIONS AND RENOVATIONS (Itemize by category)	$0
OTHER EXPENSES (Itemize by category)	$0
	$0
SUBTOTAL DIRECT COST FOR INITIAL BUDGET PERIOD	$62,000
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS	$27,900
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page)	**$89,900**
SBIR/STTR Only: FIXED FEE REQUESTED	

BUDGET FOR THE ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

ProGenetics LLC

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		0	0	0	0	0
CONSULTANT COSTS		0	0	0	0	
EQUIPMENT		0	0	0	0	0
SUPPLIES		62,000	63,860	65,776	67,749	69,782
TRAVEL		0	0	0	0	0
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		0	0	0	0	0
SUBTOTAL DIRECT COSTS		62,000	63,860	65,776	67,749	69,782
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT	62,000	63,860	65,776	67,749	69,782
	F&A	27,900	28,737	29,599	30,487	31,402
TOTAL DIRECT COSTS		89,900	92,597	95,375	98,236	101,183
TOTAL DIRECT COSTS FOR THE ENTIRE PROPOSED PROJECT PERIOD *(Item 8A, Face Page)*						$477,291
SBIR/STTR Only Fee Requested		0	0	0	0	0
SBIR/STTR Only: Total Fee Requested for Entire Proppose d Project Preiod						$0

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

PERSONNEL

Julian Cooper, Ph.D., CEO of ProGenetics LLC
Dr. Cooper is CEO of ProGenetics LLC and is providing his time to the project at no cost.

SUPPLIES COSTS

Milking: This is to cover part of the cost of maintaining and milking five lactating pigs. This cost also includes shipping the fresh frozen milk to the University of Nebraska-Lincoln. ProGenetics is providing a majority of the cost for the milk as an in-kind contribution to the project.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
William H. Velander, Ph.D.	Chair, Department of Chemical Engineering

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Illinois Benedictine College, Lisle, IL	B.S.	1977	Biochemistry
Illinois Institute of Technology, Chicago, IL	M.ChE	1980	Chemical Engineering
The Pennsylvania State University, State College, PA	Ph.D.	1987	Chemical Engineering

A. POSITIONS AND HONORS

Positions and Employment

1976-1977 Clinical Chemistry Technologist, Northwest Suburban Hospital, Arlington Heights, IL
1980-1982 Research Engineer (Drug Synthesis and Scale-up), Merck, Sharp and Dohme Research Laboratories, Merck and Co. Rahway, NJ
1986-1992 Assistant Professor, Department Chemical Engineering, Virginia Polytechnic Institute and State University (VPISU)
1992-1996 Associate Professor, Department Chemical Engineering, VPISU
1994 Visiting Scientist (Calcium-dependent Monoclonal Antibody-Protein C Interactions using Biacore), Pharmacia Biotechnology AB, Uppsala, Sweden
1997 Full Professor, Department Chemical Engineering, VPISU
1998 W. Martin Johnson Professor of Engineering, VPISU
2001 Chief Technology Officer of ProGenetics-HemoCare LLC
2002 Distinguished Lecturer, University of Mayaguez, Puerto Rico, Merck Sharp & Dohme Lecture Series
2002 Faculty Member, School of Medical and Bioengineering, VPISU
2003-present Chair, Department of Chemical Engineering, University of Nebraska-Lincoln, Lincoln, NE

Other Experience and Professional Memberships

1994-1994 Consultant, USFDA for formulation of federal regulatory guidelines for human therapeutics derived from transgenic animals
1996-2002 Co-inventor of several U.S. patents concerning gene transfer and the production of recombinant proteins of haemostatsis; all have been licensed and are in process of commercialization
2003 Advisory Board Member, Centre for Blood Research, University of British Columbia
2000-present Elected Fellow, American Institute of Medical and Bioengineering
1995-present Member, American Chemical Society

Honors

1994 Virginia Technological Institute Alumni Award for Excellence in Research
2000 Elected Fellow of the American Institute for Medical and Bioengineering

B. SELECTED PEER-REVIEWED PUBLICATIONS (in chronological order)

W.N. Drohan, D.-W. Zhang, R.K. Paleyanda, R. Chang, M. Wroble, **W. Velander** and H.K. Lubon, Inefficient Processing of Human Protein C in the Mouse Mammary Gland, *Transgenic Research*, 3:355-364, 1994.
W.L. Fodor, B.L. Williams, L.A. Matis, J.A. Madri, S.A. Rollins, J.W. Knight, **W. Velander** and S.P. Squinto, Expression of a Functional Human Complement Inhibitor in a Transgenic Pig as a Model for the Prevention of Xenogeneic Hyperacute Organ Rejection, *Proc. Natl. Acad. Sci. USA*, 91:11153-11157, 1994.

T. Morcol, R.M. Akers, J.L. Johnson, B.L. Williams, F.C. Gwazdauskas, J.W. Knight, H. Lubon, R.K. Paleyanda, W.N. Drohan and **W.H. Velander**, The Porcine Mammary Gland as a Bioreactor for Complex Proteins, *The Annals of the New York Academy of Sciences*, 721, 218-233, 1994.

R.L. Page, S.P. Butler, A. Subramanian, F.C. Gwazdauskas, J.L. Johnson, and **W.H. Velander**, Transgenesis in Mice by Cytoplasmic Injection of Polylysine/DNA Mixtures, *Transgenic Research*, 4:353-360, 1995.

H. Lubon, R. K. Paleyanda, **W. H. Velander** and W. N. Drohan, Blood Proteins from Transgenic Animal Bioreactors, *Transfusion Medicine Reviews*, 10:131-143, 1996.

T. K. Lee, N. Bangalore, **W. Velander**, W. N. Drohan and H. Lubon, Activation of Recombinant Human Protein C, *Thrombosis Research*, 82:225-234, 1996.

A. Subramanian, R. K. Paleyanda, H. Lubon, B. L. Williams, F. C. Gwazdauskas, J. W. Knight, W. N. Drohan and **W. H. Velander**, Rate Limitations in Posttranslational Processing by the Mammary Gland of Transgenic Animals, *The Annals of the New York Academy of Sciences*, 782, 87-96, 1996.

S. P. Butler, K. Van Cott, A. Subramanian, F.C. Gwazdauskas and **W. H. Velander**, Current Progress in the Production of Recombinant Human Fibrinogen in the Milk of Transgenic Animals, *Thrombosis and Haemostasis*, 78, 1:537-542, 1997.

R. K. Paleyanda, **W. H. Velander**, T. K. Lee, D. H. Scandella, F. C. Gwazdauskas, J. W. Knight, L. W. Hoyer, W. N. Drohan and H. Lubon, Transgenic Pigs Produce Functional Human Factor VIII in Milk, *Nature Biotechnology*, 15:971-975, 1997.

K. E. Van Cott, H. Lubon, C. G. Russell, S. P. Butler, F. C. Gwazdauskas, J. Knight, W. N. Drohan and **W. H. Velander**, Phenotypic and Genotypic Stability of Multiple Lines of Transgenic Pigs Expressing Recombinant Human Protein C, *Transgenic Research*, 6:1-10, 1997.

K. E. Van Cott, B. L. Williams, F. Gwazdauskas, T. Lee, H. Lubon, W. N. Drohan and **W. H. Velander**, Affinity Purification of Biologically Active and Inactive Forms of Recombinant Human Protein C Produced in the Porcine Mammary Gland, *Journal of Molecular Recognition*, 9:407-414, 1997.

W. H. Velander, H. Lubon, and W. Drohan, Transgenic Livestock as Drug Factories, *Scientific American*, 276:54-58, 1997.

A. Degener, M. Belew and **W. H. Velander**, AZn^{2+}-selective Purification of Recombinant Proteins from the Milk of Transgenic Animals, *Journal of Chromatography A*, 799:125-137, 1998.

K. Van Cott, and **W. H. Velander**, Transgenic Animals as Drug Factories: A New Source of Recombinant Protein Therapeutics. *Expert Opinion on Investigational Drugs*, 7:1683-1690, 1998.

K. Van Cott, S. P. Butler, C. G. Russell, A. Subramanian, H. Lubon, F. C. Gwazdauskas, J. Knight, W. N. Drohan, and **W. H. Velander**. Transgenic Pigs as Bioreactors: A Comparison of Gamma-carboxylation of Glutamic Acid in Recombinant Human Protein C and Factor IX by the Mammary Gland. *Genet Anal*, 15:15560, 1999.

K. E. Van Cott, H. Lubon, F. C. Gwazdauskas, J. Knight, W. N. Drohan, **W. H. Velander**, Recombinant Human Protein C Expression in the Milk of Transgenic Pigs and the Effect on Endogenous Milk Immunoglobulin and Transferrin Levels. *Transgenic Research*, 10:43-51, 2001.

W. Velander and K. Van Cott, Protein Expression Using Transgenic Animals: in *Handbook of Industrial Cell Culture Mammalian, Microbial, and Plant Cells*, eds. Vinci, Victor A. and Parekh, Sarad R. , Human Press, Totawa, NJ, 2002.

L. C. Bolling, R. S. Pleasant, S. P. Butler, **W. H. Velander,** and F. C. Gwazdauskas. An Evaluation of Sperm Mediated Gene Transfer, *Transgenics* 4:77-86, 2003.

M. Lindsay, G. C. Gil, A. Cadiz, C. Zhang, **W. H. Velander**, K. E. Van Cott. Purification of Recombinant DNA-derived Factor IX and Fractionation of Active and Inactive Subpopulations. *Journal of Chromatography*, 1026:149-157, 2004.

C. RESEARCH SUPPORT

Dr. Velander has more than 17 years experience and cumulatively more than $6 million of research support in directing transgenic animal engineering research and plasma protein purification research.

<u>Ongoing Research Support</u>

None.

Completed Research Support

12/31/99-12/31/00

American Red Cross.
Production of Recombinant Fibrinogen in the Milk of Transgenic Animals
Overall project goal: Produce recombinant fibrinogen and Factor VIII in the milk of transgenic animals.
Role: Principal Investigator

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Stephan B. Abramson, Ph.D.	Principal, LifeSci Partners

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
California Institute of Technology	B.S.	1967	Chemistry
Harvard University	Ph.D.	1975	Biochemistry
University of California, Los Angeles	Post-doc	1975-1977	Protein Biochemistry
University of Southern California	Post-doc	1977-1981	Protein Biochemistry

A. POSITIONS AND HONORS

Positions and Employment

1981-1986 Investigator (Biochemistry and Cell Biology), Huntington Medical Research Institutes, Pasadena, CA
1986 Clinical Research Associate, Alpha Therapeutic Corporation, Los Angeles, CA
1986-1989 Clinical Project Manager, Alpha Therapeutic Corporation, Los Angeles, CA
1989-1992 Associate Director, Clinical Research, Alpha Therapeutic Corporation, Los Angeles, CA
1991-2002 Associate Director, Business Development, Alpha Therapeutic Corporation, Los Angeles, CA
2001-2002 Associate Director, Clinical Affairs, Alpha Therapeutic Corporation, Los Angeles, CA
2002-2003 Director, Corporate and Business Development, Alpha Therapeutic Corporation, Los Angeles, CA
2002-2003 Director, Clinical Affairs, Alpha Therapeutic Corporation, Los Angeles, CA
2003-present Principal, LifeSci Partners, Altadena, CA

Other Experience and Professional Memberships

Professional Experience:

- Extensive industrial experience in clinical development, business development, and corporate project management in the development and commercialization of plasma-derived protein therapeutics, including especially coagulation factors (one of them a high-purity Factor IX) and intravenous immune globulin (IgG).
- Preparation and submission of 18 IND submissions, with more than 25 clinical protocols and more than 20 final clinical reports, along with other submissions that led to 5 new product approvals and 4 license amendments.
- Extensive experience in product licensing and the development and management of corporate alliances.
- Internal consulting at Alpha Therapeutic Corporation and with corporate partners in a variety of areas, including, quality systems development, regulatory strategy, and the design and implementation of preclinical development programs.

Other Related Experience:

In Vivo Recovery, Half-Life, Safety and Hemostatic Efficacy of Affinity Purified Factor VIII (Antihemophilic Factor). J. M. Goldsmith, C. K. Kasper, **S. B. Abramson**, P. Battacharya and P. Novak, presented at the 1991 Annual Meeting of the National Hemophilia Foundation.

In Vivo Recovery, Half-Life and Safety of Affinity Purified Solvent-Detergent Coagulation Factor IX. C. K. Kasper, **S. B. Abramson**, J. C. Goldsmith and S. Herring, presented at the 1992 Annual Meeting of the American Society for Hematology.

Safety, in Vivo Recovery and Half-Life of Alpha-8® HP an Affinity Chromatography Purified, Solvent/ Detergent Treated Factor VIII Concentrate. Y. Arkel, **S. Abramson**, P. Bhattacharya, C. K. Kasper and A. D. Retzios, presented, XXth International Congress, World Federation of Hemophilia, 1992.

In Vivo Recovery, Half-Life, Thrombogenic Potential and Safety of Affinity Purified Solvent-Detergent Treated Coagulation Factor IX. C. K. Kasper, **S. B. Abramson**, J. C. Goldsmith and S. Herring, presented at the XXth International Congress of the World Federation of Hemophilia, 1992.

Memberships:
American Association for Advancement of Sciences, American Chemical Society, American Society for Cell Biology, American Society for Microbiology, Association of University Technology Managers, Licensing Executives Society (USA and Canada), New York Academy of Sciences, Society of Sigma Xi.

Reviewer:
Biochemical Pharmacology, *Blood*, *Clinical Immunology and Immunopathology*, *Experimental Cell Research*, *European Journal of Cell Biology*, *Journal of Clinical Investigation*, *Journal of Immunology* and the *Journal of Leukocyte Biology*.

Honors
Listed in American Men and Women in Science.

B. SELECTED PEER-REVIEWED PUBLICATIONS (In chronological order)

I. G. Renner, **S. B. Abramson** and A. P. Douglas. Alpha-Amylase of Human Pure Pancreatic Juice: Effects of Pancreatic Disease and the Occurrence of Variant Forms in Pancreatic Juice from Healthy Volunteers. *Clin Chim Acta* 99**:**259-266, 1979.

H. Rinderknecht, N. F. Adham, I. G. Renner and **S. B. Abramson**. Diagnosis of Pancreatic Disease by Fluorometric Determination of Plasma Trypsinogen. *Prog Clin Enzymol* 2, 1981.

S. B. Abramson, H. Rinderknecht and I. G. Renner. Ribonuclease C and Pancreatic Secretory Proteins in the Peripheral Circulation before and after Pancreatectomy for Pancreatic Cancer. *Digest Dis Sci* 27:889-896, 1982.

R. Carmel, **S. B. Abramson** and I. G. Renner. Characterization of Human Pure Pancreatic Juice: Cobalamin Content, Cobalamin-Binding Proteins and Activity against Human R Binders of Various Secretions. *Clin Sci* 64*:*193-205, 1983.

J. L. Pantoja, I. G. Renner, **S. B. Abramson** and H. A. Edmundson. Production of Acute Hemorrhagic Pancreatitis in the Dog using Venom of the Scorpion Buthus Quinquestriatus. *Digest Dis Sci* 28:429-439, 1983.

I. G. Renner, J. L. Pantoja, **S. B. Abramson**, F. E. Russell and M. M. Koch. Effects of Scorpion and Rattlesnake Venoms on the Canine Pancreas Following Pancreaticoduodenal Arterial Injections. *Toxicon* 21:405-420, 1983.

H. Rinderknecht, I. G. Renner, **S. B. Abramson** and C. Carmack. Mesotrypsin: A New Inhibitor-Resistant Protease from a Zymogen in Human Pure Pancreatic Juice. *Gastroenterology* 86:681-692, 1984.

W. F. Agnew, R. B. Alvarez, T. G. H. Yuen, **S. B. Abramson** and D. Kirk. A Serum-Free Culture System for Studying Solute Exchanges in the Choroid Plexus. *In Vitro* 20:712-722, 1985.

R. Carmel, D. Hollander, H. M. Gergely, I. G. Renner and **S. B. Abramson**. Pure Human Pancreatic Juice Directly Enhances Uptake of Cobalamin by Guinea Pig Ileum in Vivo. *Proc Soc Exp Biol Med Sci* 178:143-150, 1985.

R. T. Chlebowski, **S. B. Abramson**, J. R. Bateman, J. M. Weiner and I. G. Renner. Influence of Nutritional Status on Circulatory Ribonuclease C Levels in Patients with Cancer. *Cancer* 55:427-431, 1985.

A. Khwaja, B. Johnson, I. E. Addison, **S. B. Abramson**, K. Ruthven, K. Yong and D. C. Linch. In Vivo Effects of Macrophage Colony-Simulating Factor on Human Monocyte Function. *Br J Haematol* 77:25-31, 1991.

A. Khwaja, K. Yong, H. M. Jones, R. Chopra, A. K. McMillan, A. H. Goldstone, K. G. Patterson, C. Matheson, K. Ruthven, **S. B. Abramson** and D. C. Linch. The Effect of Macrophage Colony-Stimulating Factor on Haemopoietic Recovery after Autologous Bone Marrow Transplantation. *Br J Haematol* 81:288-295, 1992.

C. RESEARCH SUPPORT

Ongoing Research Support

Not Applicable

Completed Research Support

Not Applicable

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Julian D. Cooper, Ph.D.	President and Chief Executive Officer

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Bradford University, UK	B.Tech	1979	Medical Sciences
Birmingham University, UK	M.Sc	1982	Biotechnology
Birmingham University, UK	Ph.D.	1985	Chemical Engineering
Reading University, UK	Post-Doc	1986-1989	Microbiology

A. POSITIONS AND HONORS

Positions and Employment

1979-1981 Research Technician, Interferon Laboratory, Warwick University, Coventry, England
1989-1992 Molecular Biologist, PPL Therapeutics LTD., Roslin, Scotland
1992-1994 Team Leader and Project Manager, PPL Therapeutics LTD., Roslin, Scotland
1995-1996 General Manager, PPL Therapeutics Inc., Blacksburg, VA
1997 Vice President and General Manager, PPL Therapeutics Inc., Blacksburg, VA
1997-1999 Chief Operating Officer, PPL Therapeutics Inc., Blacksburg, VA
2000-present President and Chief Executive Officer, ProGenetics LLC, Blacksburg, VA

Other Experience and Professional Memberships

None

Honors

None

B. SELECTED PEER-REVIEWED PUBLICATIONS (in chronological order)

M. Skinner, V. R. Racaniello, G. Dunn, **J. Cooper,** P.D. Minor, and J. W. Almond. New Model for the Secondary Structure of the 5' Non-coding RNA of Poliovirus is Supported by Biochemical and Genetic Data that also Show that RNA Secondary Structure is Important in Neurovirulence. *J. Mol. Biol.*, 207(2):379-392, 1989.

T. Urakawa, M. Ferguson, P. D. Minor, **J. Cooper,** M. Sullivan, J. W. Almond, and D. H. L. Bishop. Synthesis of Immunogenic but Non-infectious Poliovirus Particles in Insect Cells by a Baculovirus Expression Vector. *J. Gen. Virol*. 70(6):1453-1464, 1989.

A. Carver, G. Wright, D. Cottom, **J. Cooper,** M. Dalrymple, S. Temperley, M. Udell, D. Reeves, J. Percy, and A. Scott. Expression of Human Alpha 1 Antitrypsin in Transgenic Sheep. *Cytotechnology*, 1(3):77-84, 1992.

A. Stacey, A. Schnieke, J. McWhir, **J. Cooper,** A. Colman, and D. W. Melton. Use of Double-replacement Gene Targeting to Replace the Murine Alpha-lactalbumin Gene with its Human Counterpart in Embryonic Stem Cells and Mice. *Mol. Cell Biol.*, 14:1009-1016, 1994.

J. D. Cooper, and M. A. Dalrymple. The Use of Transgenic Sheep as Bioreactors. *Experimental Medicine, Developmental Biotechnology Suppl.*, 12(2):124-132, 1994.

J. Cooper. Protein Production in Transgenic Animals. *NABC report*, 8:137-144, 1996.

J. D. Cooper, and A. E. Schnieke. Alpha-lactalbumin Gene Constructs. US patent US5852224, 1998.

W. H. Eyestone, M. Gowallis, J. Monohan, T. Sink, S. F. Ball, and **J. D. Cooper**. Production of Transgenic Cattle Expressing Human Alpha-lactalbumin in Milk. *Theriogenology*, 49(1):386, 1998.

J. D. Cooper, W. H. Velander, and T. K. O'Sickey. Production of High Levels of Transgenic Factor IX without Gene Rescue, and its Therapeutic Uses. Patent application PCT/US02/07532.

J. D. Cooper, T. K. O'Sickey, and S. B. Butler. Transgenic Proteins from Multi-gene Systems, Methods, Compositions, Uses and the like Relating Thereto. Patent application PCT/02/07540.

J. D. Cooper, W. H. Velander, and S. P. Butler. Production of High Levels of Transgenic Factor VIII with Engineered Stability, and its therapeutic uses. Patent application PCT/US02/07530.

C. RESEARCH SUPPORT

Ongoing Research Support
None

Completed Research Support
None

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
William S. Dernell, D.V.M.	Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Harper College, Palatine, IL		1977-1978	General Science
Utah State University, Logan, UT	B.S	1978-1981	Animal Science
University of Illinois, Urbana, IL	B.S., D.V.M.	1981-1985	Veterinary Science
Washington State University, Pullman, WA	M.S.	1990-1993	Veterinary Science

A. POSITIONS AND HONORS

Positions and Employment

1986-1988 Relief State Veterinarian, Colorado Racing Commission, Denver, CO
1985-1990 Associate Veterinarian, Rio Cucharas Veterinary Clinic, Walsenburg, CO
1987-1990 Associate Veterinarian, Allpets Clinic, Boulder, CO
1990-1993 Small Animal Surgical Resident, Washington State University, Pullman, WA
1993-1994 Surgical Oncology Fellowship, Colorado State University, Ft Collins, CO
1994-1995 Oncology Research Fellowship, Colorado State University, Ft Collins, CO
1995-2001 Assistant Professor, Surgical Oncology, Colorado State University, Ft Collins, CO
2001-present Associate Professor, Surgical Oncology, Colorado State University, Ft. Collins, CO
2002-present Chief, Clinical Oncology Service, Colorado State University, Ft. Collins, CO

Other Experience and Professional Memberships

American College of Veterinary Surgeons (Diplomate Status since 1994); American Veterinary Medical Association, American Animal Hospital Association; Veterinary Cancer Society, Veterinary Orthopedic Society Veterinary Comparative Oncology Group; Phi Zeta Honor Society, American Association of Veterinary Clinicians, Colorado Veterinary Medical Association.

Honors

2001 Phi Zeta Research Day First Place Faculty Poster Presentation, Colorado State University
1998 Pfizer Animal Health Award for Research Excellence, Colorado State University
1996 Member of a Program of Research and Scholarly Excellence, Colorado State University, Fort Collins, CO
1993 Surgery Scholarship, Washington State University, College of Veterinary Medicine
1993 Phi Zeta Honor Society Member
1992 Washington State University, Travel Grant Award
1991 Dr. Ivor H. Evans Scholarship, Washington State University

B. SELECTED PEER-REVIEWED PUBLICATIONS (in chronological order)
(Publications since 1998)

S. A. Mendoza, T. Konishi, **W. S. Dernell**, S. J. Withrow and C. W. Miller. Status of the P53, Rb and Mdm2 Genes in Canine Osteosarcoma, *Anticancer Res*, 18:4449-4454, 1998.

W. S. Dernell. Treatment of Severe Orthopedic Infections, *Vet Clin North Am: Small Anim Pract*, 29:1261-1274, 1999.

N. Ehrhart, **W. S. Dernell**, E. J. Ehrhart, J. M. Hutchison, E. B. Douple, J. H. Brekke, R. C. Straw and S. J. Withrow. The Effects of a Controlled-Release Cisplatin Delivery System after Marginal Resection of Breast Carcinoma, *Am J Vet Res*, 60(11):1347-1351, 1999.

M. J. Thomson, S. J. Withrow, **W. S. Dernell** and B. E. Powers. Intermuscular Lipomas of the Thigh Region in Dogs: 11 Cases, *J Am Hosp Assoc*, 35:165-167, 1999.

V. S. Bregazzi, **W. S. Dernell**, D. J. Huber, R. Schochet, M. Lafferty, B. E. Powers, G. K. Ogilvie, S. J. Withrow and M. C. Manning. Intracavitary Slow Release Paclitaxel Polymer in a Rodent Model of Mammary Carcinoma, *J Bioact Compat Polymer*, 15:85-88, 2000.

W. S. Dernell, B. J. Van Vechten, R. C. Straw, S. M. LaRue, B. E. Powers and S. J. Withrow. Outcome Following Treatment of Vertebral Tumors in 20 Dogs (1986-1995), *J Am Anim Hosp Assoc*, 36:245-251, 2000.

S. E. Lana, G. K. Ogilvie, R. A. Hansen, B. E. Powers, **W. S. Dernell** and S. J. Withrow. Identification of Matrix Metalloproteinases in Canine Neoplastic Tissue, *Am J Vet Res*, 61(2):111-114, 2000.

W. S. Dernell, C. Gentry-Weeks, M. C. Manning, B. E. Powers, R. D. Park, M. Lafferty, C. A. Kuntz, J. E. Shively, R. F. Falk, J. D. Meyer, T. W. Randolph and S. J. Withrow. In Vivo Evaluation of Antibiotic Impregnated Beads in a Rat Osteomyelitis Model, *J Bioact Compat Polymer*, 16:235-250, 2001.

W. S. Dernell, S. J. Withrow, C. A. Kuntz, R. Dewell, F. B. Garry, B. E. Powers, J. E. Shively, R. F. Falk, J. D. Meyer, M. C. Manning and T. W. Randolph. In Vivo Evaluation of Gentamicin Impregnated Polylactic Acid Beads Implanted in Sheep, *J Bioact Compat Polymer*, 16:119-135, 2001.

S. D. Ferreira, **W. S. Dernell**, B. E. Powers, R. A. Schochet, D. J. Huber, C. A. Kuntz, S. J. Withrow, R. M. Wilkins and M. Jackson. Effects of gas plasma sterilization on demineralized bone matrix, *Clin Orthop Rel Res*, 388:233-239, 2001.

N. Leibman, C. Kuntz, P. Steyn, M. Fettman, B. Powers, S. Withrow and **W. Dernell**. The Measurement of the Proximal Extent of Canine Osteosarcoma of the Distal Radius Using Radiography, Nuclear Scintigraphy, and Histopathology, *Vet Surg*, 30:240-245, 2001.

V. S. Bregazzi, S. M. LaRue, E. McNiel, D. W. Macy, **W. S. Dernell**, B. E. Powers and S. J. Withrow. Treatment with a Combination of Doxorubicin, Surgery and Radiation Versus Surgery and Radiation Alone for Cats with Vaccine-Associated Sarcomas: 25 Cases (1995-2000), *J Am Vet Med Assoc.*, 218(4):547-550, 2001.

M. L. Mehl, S. J. Withrow, B. Seguin, B. E. Powers, **W. S. Dernell**, A. D. Pardo, R. C. Rosenthal, S. Z. Dolginow and R. D. Park. Spontaneous Regression of Osteosarcoma in Four Dogs. *J Am Vet Med Assoc*, 219(5):614-617, 2001.

B. Seguin, N. F. Leibman, V. S. Bregazzi, G. K. Ogilvie, B. E. Powers, **W. S. Dernell**, M . J. Fettman and S. J. Withrow. Clinical Outcome of Grade II Mast Cell Tumors Treated with Surgery Alone in Dogs: 55 Cases (1996-1999), *J Am Vet Med Assoc*, 218(7):1120-1123, 2001.

W. S. Dernell, B. E. Powers, D. J. Taatjes, P. Cogan, G. Gaudiano and T. H. Koch. Evaluation of Epidoxorubicin-formaldehyde Conjugate, Epidoxoform in a Mouse Mammary Carcinoma Model, *Cancer Invest*, 20:713-724, 2002.

E. Morello, **W. S. Dernell**, C. A. Kuntz, S. M. LaRue, M. Lafferty, A. Nelson, J. H. Brekke, C. H. Mallinckrodt, S. J. Withrow and M. C. Manning. Evaluation of Cisplatin in Combination with a Biologic Response Modifier in a Murine Mammary Carcinoma Model, *Cancer Invest*, 204(4):480-489, 2002.

B. D. X. Lascelles, E. Monnet, J. M. Liptak, J. Johnson and **W. S. Dernell**. Surgical Treatment of Right-sided Renal Lymphoma with Invasion of the Vena Cava, *J Sm Anim Pract*, 44:1-4, 2003.

B. C. X. Lacelles, M. J. Thomson, **W. S. Dernell**, R. C. Straw, M. Lafferty and S. J. Withrow. Combined Dorsolateral and Intraoral Approach for the Resection of Tumors of the Maxilla in the Dog, *J Am Anim Hosp Assoc,* 39:294-305, 2003.

B. Seguin, P. J. Walsh, D. R. Mason, E. R. Wiser, J. L. Parmenter and **W. S. Dernell**. Use of an Ipsilateral Vascularized Ulnar Transposition Autograft for Limb Sparing Surgery of the Distal Radius in Dogs: An Anatomic and Clinical Study, *Vet Surg*, 32:69-79, 2003.

C. RESEARCH SUPPORT

Ongoing Research Support

DAMD17-02-1-0348 and DAMD17-02-1-0347 05/14/02-06/13/05
U.S. Army Medical Research and Material Command
Evaluation of Intracavitary Chemotherapy Delivery for Treatment of Mammary Carcinoma
Overall project goals: The major goals of this clinical bridge and career enhancement grant are to evaluate local delivery of taxol chemotherapy to control human breast tumor in nude mice.
Role: PI

1-49103 07/01/03-06/30/04
College Research Council
Antitumor Effect of a Novel Controlled Release Cisplatin Delivery System to Human Ovarian Xenografted Nude Mice
Overall project goal: The major goal of this project is to evaluate a unique chemotherapy delivery system in a rodent model of ovarian cancer.
Role: PI

RO1 CA 092107-03 06/01/01-05/31/04
NIH (subcontract)
New Drugs Targeted to Metastatic Cancer and Angiogenesis
Overall project goals: The major goals of this project are to evaluate a novel, targeted antimetastatic drug in a mouse mammary carcinoma model.
Role: PI (subcontract)

Completed Research Support

IR43CA88585-01A2 09/01/02-08/31/03
NIH (Subcontract)
Development of a Multidepot Polymer Drug Delivery System
Overall project goals: The major goals of this project are to evaluate tolerability and pharmacokinetics of a polymer system loaded with two chemotherapy drugs in small and large animal systems.
Role: PI (subcontract)

1-49113 07/01/02-07/01/03
College Research Council
Evaluation of an Active Silver Antisepsis Device in a Rodent Infection Model
Overall project goal: The major goal of this project was to evaluate a novel activated silver device for it's efficacy in treating a rat model of osteomyelitis.
Role: PI

5-35559 06/02/00-12/01/02
AlloSource
Quantification of Bone Induction by Porous and Dense Hydroxyapatite Beads in Combination with Demineralized Bone Matrix
Overall project goal: The major goal of this project was to evaluate bone induction properties of two types of hydroxyapatite beads mixed with demineralized bone matrix in a rat osteoinduction model.
R*ole*: PI

5-35167 12/01/01-11/30/02
Irish Wolfhound Club of America
Bisphosphonate Treatment for Osteosarcoma
Overall project goal: The major goal of this study is to evaluate the efficacy of aledronate (an oral bisphosphonate) against canine osteosarcoma.
Role: PI

DAMD17-01-1-0046 04/01/01-6/30/02
U.S. Army Medical Research and Materiel Command (subcontract)
Design, Synthesis and Evaluation of Activated Doxorubicin Targeted to Advanced Prostate Cancer
Overall project goals: The major goals of this project are to evaluate activated doxorubicin targeted to advanced prostate cancer in a mouse model.
Role: PI (subcontract)

5-30149 07/01/00-6/30/01
CIRB
Evaluation of Slow Release Taxol Chemotherapy in a Rodent Model for Mammary Carcinoma
Overall project goal: The major goal of this project was to evaluate the local and systemic toxicity as well as anti-tumor efficacy of a locally delivered taxol/polymer system in mice.
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Mark C. Manning, Ph.D.	Chief Technical Officer, HTD BioSystems Associate Professor, Colorado State University

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Hope College, Holland, MI	A.B.	1978	Chemistry
Northwestern University, Evanston, IL	Ph.D.	1983	Inorganic Chemistry
Colorado St. Univ., Ft. Collins, CO	Postdoc	1982-1984	Organometallic Chemistry
Colorado St. Univ., Ft. Collins, CO	Postdoc	1984-1988	Biophysical Chemistry

A. POSITIONS AND HONORS

Positions and Employment

1988-1990 Assistant Professor of Pharmaceutical Chemistry, University of Kansas
1988-1990 Courtesy Assistant Professor of Biochemistry, University of Kansas
1989 Summer Visiting Professor, Abbott Laboratories, N. Chicago, IL
1990-1997 Assistant Professor of Pharmaceutics, University of Colorado
1997-2003 Associate Professor of Pharmaceutics, University of Colorado
1997-2003 Co-Director, Center for Pharmaceutical Biotechnology, University of Colorado
2003-present Chief Technical Officer, HTD BioSystems
2003-present Special Associate Professor, Colorado State University Animal Cancer Center

Other Experience and Professional Memberships

Editorial Board for *Current Pharmaceutical Biotechnology* and *Journal of Pharmaceutical and Biomedical Analysis*; Co-organizer of the Colorado Protein Stability Conferences and the Colorado Macromolecular Delivery Conferences.

Honors

1997 Research Achievement Award in Biotechnology from the American Association of Pharmaceutical Scientists
1997 Invited Guest, Novo Nordisk Technology Conference on Protein Stability, Hvidøre, Denmark (one of only five outside scientists invited)

B. SELECTED PEER-REVIEWED PUBLICATIONS (in chronological order)
(70 publications before 2000)

S.D. Allison, A. Williams, K. Middleton, T.W. Randolph, **M.C. Manning** and J.F. Carpenter. Optimizing Storage Stability of Lyophilized Actin with Dextran/Disaccharide Mixtures, *J. Pharm. Sci.*, 89:199-214, 2000.

D. J. Claffey, J. D. Meyer, R. Beauvais, T. Brandt, E. Shefter, D. J. Kroll, J. A. Ruth, and **M. C. Manning**. Long Chain Arginine Esters: A New Class of Biocompatible Cationic Detergents, *Biochem. Cell Biol.* 78:59-65, 2000.

Y-S. Kim, J. S. Wall, J. Meyer, C. Murphy, T. W. Randolph, **M. C. Manning**, and J. F. Carpenter. Thermodynamic Modulation of Light Chain Amyloid Fibril Formation, *J. Biol. Chem.*, 275:1570-1573, 2000.

V.S. Bregazzi, W.S. Dernell, D.J. Huber, R. Schochet, M. Lafferty, G.K. Oglivie, S.J. Withrow, B.E. Powers, and **M. Manning.** Intracavitary Slow Release Paclitaxel Polymer in a Rodent Model of Mammary Carcinoma, *J. Bioactive Compatible Polym.* 15:85-88, 2000.

J-J. Lin, J. D. Meyer, J. F. Carpenter, and **M. C. Manning**. Stability of Human Serum Albumin during Bioprocessing. Denaturation and Aggregation during Processing of Albumin Paste, *Pharmaceutical Res.* 17:391-386, 2000.

T-H. Yang, J. L. Cleland, J. D. Meyer, L. S. Jones, T. W. Randolph, **M. C. Manning**, and J. F. Carpenter. Effect of Zinc-Binding and Precipitation on Structures of Recombinant Human Growth Hormone and Nerve Growth Factor, *J. Pharm. Sci.* 89:1480-1485, 2000.

A. Dong, J. D. Meyer, **M. C. Manning**, J. L. Brown, and J. F. Carpenter. Comparative FT-IR and CD Spectroscopic Analysis of ∀1-Proteinase Inhibitor and Ovalbumin in Aqueous Solution, *Arch. Biochem. Biophys.* 383:148-155, 2000.

W.S. Dernell, S.J. Withrow, **M.C. Manning**, C.A. Kuntz, R. Dewell, F.B. Garry, B.E. Powers, J.E. Shively, R.F. Falk, and T.W. Randolph. In Vivo Evaluation of Gentamicin-Impregnated Polylactic Acid Beads Implanted in Sheep, *J. Bioactive Compatible Polym* 16:119-135, 2001.

Y-S. Kim, S. P. Cape, E. Chi, R. Raffen, P. Wilkins-Stevens, F. J. Stevens, **M. C. Manning**, T. W. Randolph, A. Solomon, and J. F. Carpenter. Counteracting Effects of Renal Solutes in Amyloid Fibril Formation by Immunoglobulin Light Chains, *J. Biol. Chem.* 276:1626-1633, 2001.

E. Morello, W. S. Dernell, C. A. Kuntz, M. Lafferty, A. Nelson, J. H. Brekke, C. H. Mallinckrodt, S. J. Withrow, and **M. C. Manning**. Evaluation of Cisplatin in Combination with a Biological Response Modifier in a Murine Mammary Carcinoma Model, *Cancer Investigations* 20:480-489, 2002.

R. Nayar and **M. C. Manning**. High Throughput Development: Meeting the Coming Challenges, *BioPharm* Feb, 25-32, 2002.

H. Zhou, C. S. Lengsfeld, D. J. Claffey, J. A. Ruth, B. A. Hybertson, T. W. Randolph, K-Y. Ng, and **M. C. Manning**. Ion Pairing of Isoniazid Using a Prodrug Approach, *J. Pharm. Sci.* 91:1502-1511, 2002.

S. Krishnan, E. Y. Chi, J. N. Webb, B. S. Chang, D. Shan, M. Goldenberg, **M. C. Manning**, T. W. Randolph, and J. F. Carpenter. Aggregation of Granulocyte Colony Stimulating Factor under Physiological Conditions: Characterization and Thermodynamic Inhibition, *Biochemistry* 41:6422-6431, 2002.

C. S. Lengsfeld, **M. C. Manning**, and T. W. Randolph. Encapsulating DNA within Biodegradable Polymeric Microspheres, *Curr. Pharm. Biotechnol.* 3:227-235, 2002.

D. Pitera, C. S. Lengsfeld, **M. C. Manning**, and T. W. Randolph. Dissolution and Partitioning Behavior of Hydrophobic Ion Paired Complexes, *Pharm. Res.* 19:1572-1576, 2002.

J. D. Meyer, **M. C. Manning**, and D. G. Vander Velde. Characterization of the Solution Conformations of Leuprolide Acetate, *J. Peptide Res.* 60:159-168, 2002.

R. Krishnamurthy and **M. C. Manning**. The Stability Factor: Importance in Formulation Development, *Curr. Pharm. Biotechnol*. 3:361-371, 2002.

M. M. Tomczak, L. Vigh, J. D. Meyer, **M. C. Manning**, D. K. Hincha, and J. H. Crowe. Lipid Unsaturation Determines the Interaction of AFP Type I with Model Membranes during Thermotropic Phase Transitions, *Cryobiology* 45:135-142, 2002.

Y.-S. Kim, T. W. Randolph, **M. C. Manning**, F. J. Stevens, and J. F. Carpenter. Congo Red Populates Partially Unfolded States of an Amyloidogenic Protein to Enhance Aggregation and Amyloid Fibril Formation, *J. Biol. Chem.* 278:10842-10850, 2003.

Y. Zhang, W. Garzon-Rodriguez, **M. C. Manning**, and T. J. Anchordoquy. The Use of Fluorescence Resonance Energy Transfer to Monitor Dynamic Changes of Lipid-DNA Interactions during Lipoplex Formation *Biochim. Biophys. Acta* 1614:182-192, 2003.

C. RESEARCH SUPPORT

Ongoing Research Support

T32 CA79446 07/01-06/06
NIH
Leadership Training in Pharmaceutical Biotechnology
Overall project goal: This grant provides graduate student support for the Center's training programs
Role: Co-PI (T. Randolph, PI)

Completed Research Support

R01 AI47446 04/99-03/03
NIH
Aerosol-Based Nanoparticle Drug Delivery System for Tuberculosis
Overall project goal: This grants supports work on the development of inhaled controlled release treatments for TB
Role: Collaborator (PI Lawrence Ng)

03/02-02/03
HTD Biosystems
Studies on Novel Protein Formulations
Overall project goal: This contract supported studies on the stability of novel protein formulations
Role: PI

02/02-01/03
Human Genome Sciences
Characterization of Novel Protein Hormone
Overall project goal: This grant supports work on the interaction of a novel cytokine with excipients.
Role: PI

08/01-07/02
GonexPets
Preformulation of Toxin-Gonadotropin Conjugate
Overall project goal: This grant covers studies on the stability and structure of a toxin-gonadotropin conjugate
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Michael M. Meagher, Ph.D.	Professor and Director of the UNL Biological Process Development Facility

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Colorado State University, Fort Collins, CO	B.S.	1981	Engineering Science
Iowa State University, Ames, IA	M.S.	1984	Chemical Engineering
Iowa State University, Ames, IA	Ph.D.	1987	Chemical Engineering

C. POSITIONS AND HONORS

Positions and Employment

1981–1987 Graduate Research Assistant, Iowa State University
1987–1989 Senior Scientist/Biochemical Engineer, Hoffmann-LaRoche
1989–1995 Assistant Professor, University of Nebraska-Lincoln
1995–2003 Associate Professor, University of Nebraska-Lincoln
2003–present Professor, Donald and Mildred Othmer Endowed Chair, University of Nebraska-Lincoln

Other Experience and Professional Memberships

Member, American Institute of Chemical Engineering and American Chemical Society

D. SELECTED PEER-REVIEWED PUBLICATIONS (in chronological order)

V. Chiruvolu, J. M. Cregg, and **M. M. Meagher**. Recombinant Protein Production in an Alcohol Oxidase-defective Strain of *Pichia pastoris* in Fed-batch Fermentations. *Enzyme Microbiol. Technol*. 21:277-283, 1997.

V. Chiruvolu, K. Eskridge, J. Cregg, and **M. Meagher**. Effects of Glycerol Concentration and pH on Growth of Recombinant *Pichia pastoris* Yeast. *Appl. Biochem. Biotechnol*. 75:163-173, 1998.

K.J. Potter, M. A. Bevins, E. V. Vassilieva, V. J. Chiruvolu, T. Smith, L. A. Smith, **M. M. Meagher**. Production and Purification of the Heavy-chain Fragment C of Botulinum Neurotoxin, Serotype B, Expressed in the Methylotropic Yeast *Pichia pastoris*. *Protein Express. Purif*. 13:357-365, 1998.

K.J. Potter, W. Zhang, L.A. Smith, and **M.M. Meagher**. Production and Purification of the Heavy Chain Fragment C of Botulinum Neurotoxin, Serotype A, Expressed in the Methylotropic Yeast *Pichia pastoris*. *Protein Express. Purif*. 19:393-402, 2000.

W. Zhang, M.A. Bevins, B.A. Plantz, L.A. Smith, and **M.M. Meagher**. Modeling *Pichia pastoris* Growth on Methanol and Optimizing the Production of a Recombinant Protein, the Heavy-Chain Fragment C of Botulinum Neurotoxin, Serotype A. *Biotechnol. Bioeng*. 70(1):1-8, 2000.

W. Zhang, M. Inan and **M. M. Meagher**. Fermentation Strategies for Recombinant Protein Expression in the Methylotrophic Yeast *Pichia pastoris*. *Biotechnol. Bioprocess Eng*. 5(4):275-287, 2000.

M. Inan and **M. Meagher**. Non-Repressing Carbon Sources for Alcohol Oxidase (AOX1) Promoter of *Pichia pastoris. J. Biosci. Bioeng*. 92:585-589, 2001.

M. Inan and **M. Meagher**. The Effect of Ethanol and Acetate on Protein Expression in *Pichia pastoris*. *J. Biosci. Bioeng*. 92:337-341, 2001.

W. Zhang, L. A. Smith, B. A. Plantz, V. L. Schlegel, and **M. Meagher**. Design of Methanol Feed Control in *Pichia pastoris* Fermentation Based Upon a Growth Model. *Biotechnol. Prog*. 18(6):1392-1399, 2002.

B. A. Plantz, J. Andersen, L. A. Smith, **M. M. Meagher**, V. L. Schlegel. Detection of Non-Host Viable Contaminants in *Pichia pastoris* Cultures and Fermentation Broths. *J. Indust. Microbiol. Biotechnol*. 30(11):643-650, 2003.

S. K. Johnson, W. Zhang, L. A. Smith, K. J. Hywood-Potter, T. S. Swanson, V. L. Schlegel, **M. Meagher**. Scale-up of the Fermentation and Purification of the Recombinant Heavy Chain Fragment C of Botulinum Neurotoxin Serotype F Expressed in *Pichia pastoris.* *Protein Express. Purif.* 32(1):1-9, 2003.

W. Zhang, K. J. Hywood Potter, B. A. Plantz, V. L. Schlegel, L. A. Smith, **M. Meagher**. *Pichia pastoris* Fermentation with Mixed-Feeds of Glycerol and Methanol: Growth Kinetics and Production Improvement. *J. Industr.. Microbiol. Biotechnol.* 30(4):210-215, 2003.

J. Sinha, B. A. Plantz, W. Zhang, M. Gouthro, V. L. Schlegel, C.-P. Liu, **M. Meagher**. Improved Production of Recombinant Ovine Interferon-τ by Mut$^+$ Strain of *Pichia pastoris* Using an Optimized Methanol Feed Profile. *Biotechnol. Prog.* 19(3):794-802, 2003.

C. RESEARCH SUPPORT

Ongoing Research Support

U01-AI056514-01 09/02/03 – 09/01/07
NIH
Fast Track Development of a Heptavalent Vaccine Against the Botulinum Neurotoxin
Overall project goal: The goal of this project is to complete all aspects of process development for a recombinant vaccine against the botulinum neurotoxin. This includes molecular biology, fermentation, purification, analytical methods development, and technology transfer for cGMP production of serotypes C, D, E, F, and G of the botulinum neurotoxin.
Role: Co-PI

DAMD17-02-1-0659 03/15/02-03/14/05
UAMRMC
Process Research and Development of Antibodies as Countermeasures for *C. botulinum*
Overall Project Goal: The goal of this project is to express and optimize expression in CHO cells of human antibodies against the the botulinum neurotoxin. The goal of the this project is to fill out laboratory space on third floor of Othmer Hall, new home for the UNL Biological Process Development Facility
Role: PI

DAMD17-02-C-0107 01/01/02 – 01/01/05
USAMRMC
Process Research and Development for Therapeutic Agents and Vaccines as Countermeasures Against Biological Warfare Agents
Overall project goal: The goal of this project is the research and development of processes to produce vaccines and therapeutic agents against biological threats. The Army needs countermeasures to protect combat and non-combat personnel against biological threats.
Role: PI

Completed Research Support

DAMD17-98-C8034 01/01/98 – 12/30/01
USAMRMC
Fermentation Process Development for the Production of the Hc Fragment of the Botulinum Neurotoxin Serotype A
Overall project goal: This is part of the project "Fermentation, Recovery, and Purification of the Hc Fragment of the Botulinum Neurotoxin from *Pichia pastoris*" contracted with USAMRMC. The goal is to develop a workable and scalable fermentation and purification process research and development of botulinum vaccine candidates.
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Paul E. Monahan, M.D.	Assistant Professor, Pediatric Hematology/Oncology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Princeton University, Princeton, NJ	B.A.	1986	English
University of Virginia School of Medicine, Charlottesville, VA	M.D.	1990	Medicine
University of North Carolina, Chapel Hill, NC	Internship/ Residency	1990-1993	Pediatrics
University of North Carolina, Chapel Hill, NC	Fellow	1993-1998	Pediatric Hematology and Oncology
University of North Carolina School of Medicine	Post-doc	1994-1998	Adeno-associated virus (AAV) delivery of factor IX gene therapy
National Hemophilia Foundation	Postgraduate Research Fellow	1998-2000	AAV factor IX gene delivery to muscle

A. POSITIONS AND HONORS

Positions and Employment

1981-1982 Nursing Assistant, General Medicine and Post-anesthesia Care units, Winchester Medical Center, Winchester, VA
1982-1983 Psychiatric Technician, Winchester Medical Center, Winchester, VA
1998-2000 Research Assistant Professor, Department of Pediatrics, Division of Hematology/Oncology, University of North Carolina, Chapel Hill, NC
2001-present Assistant Professor, Department of Pediatrics, Division of Hematology/Oncology, Attending Physician, Comprehensive Hemophilia Diagnostic and Treatment Center, University of North Carolina, Chapel Hill, NC

Other Experience and Professional Memberships

1997-present American Society of Hematology: Member
1998-present Children's Oncology Group: Member
1999-present International Society of Thrombosis and Hemostasis: Member
1999-present Hemophilia Research Society: Member
2001-present American Society of Gene Therapy: Member
1994-2000 American Academy of Pediatrics: Member
2003-present Editorial Board, Hematology Section, *The Annals of Pharmacotherapy*

Specialty Certification

October 1995 American Board of Pediatrics: Board Certified in General Pediatrics
November 1996;
Renewed July 2002 American Board of Pediatrics: Board Certified in Pediatric Hematology and Oncology

Medical Licensure

1996-present North Carolina Medical Board

Honors

July 1996-1998	NIH NIDDK Individual National Research Service Award (NRSA), Packaging Cell Lines for Adeno-associated Virus Vectors. Mentor: R. Jude Samulski, Ph.D.
July 1998-July 2000	National Hemophilia Foundation Judith Graham Pool Postgraduate Research Fellowship Award, AAV Hemophilia Gene Therapy Vectors

B. SELECTED PEER-REVIEWED PUBLICATIONS (in chronological order)

J. R. Kerrigan, P. M. Martha Jr., R. J. Krieg Jr., T. A.Queen, **P. E. Monahan**, and A. D. Rogol. Augmented Hypothalamic Proopiomelanocortin Gene Expression with Pubertal Development in the Male Rat: Evidence for an Androgen Receptor-Independent Action. *Endocrinology*, 128:1029-103, 1991.

P. E. Monahan, R. J. Samulski, J. Tazelaar, X. Xiao, T. C. Nichols, D. A. Bellinger, M. S. Read, and C. E. Walsh. Direct Intramuscular Injection with Recombinant AAV Vectors Results in Sustained Expression in a Dog Model of Hemophilia. *Gene Therapy*, 5(1):40-49, 1998.

H. J. Chao, R. J. Samulski, D. Bellinger, **P. E. Monahan**, T. Nichols, and C. E. Walsh. Persistent Expression of Canine Factor IX in Hemophilia B Canines. *Gene Therapy*, 6(10):1695-1704, 1999.

A. K. Malik, **P. E. Monahan**, D. L. Allen, B-G Chen, R. J. Samulski, and K. Kurachi. Kinetics of Recombinant Adeno-associated Virus (AAV)-mediated Gene Transfer. *J Virology*, 74(8):3555-3565, 2000.

P. E. Monahan and R. J. Samulski. AAV Vectors: Is Clinical Success on the Horizon? *Gene Therapy*, 7(1): 24-30, 2000.

P. E. Monahan and R. J. Samulski. Adeno-associated Virus Vectors for Gene Therapy: More Pros than Cons? *Molecular Medicine Today*, 6:433-440, 2000.

J. Blatt, S. H. Gold, J. M. Wiley, **P. E. Monahan**, H. C. Cooper, and D. Harvey. Off-label Use of Recombinant Factor VIIa in Patients Following Bone Marrow Transplantation. *Bone Marrow Transplantation*, 28(4):405-407, 2001.

H. Chao*, **P. E. Monahan***, Y. Liu, R. J. Samulski, and C. E. Walsh. Sustained and Complete Phenotype Correction of Hemophilia B Mice Following Intramuscular Injection of AAV1 Serotype Vectors. *Molecular Therapy*, 4(3):217-222, 2001. *contributed equally.

D. M. McCarty, **P. E. Monahan**, and R. J. Samulski. Self-complementary Adeno-associated Virus (scAAV) Vectors Promote Efficient Transduction Independently of DNA Synthesis. *Gene Therapy*, 8(16):1248-1253, 2001.

P. E. Monahan, K. Jooss, and M. S. Sands. Safety of Adeno-associated Virus Gene Therapy Vectors: A Current Evaluation. *Expert Opinions in Drug Safety*, 1(1):79-91, 2002.

M. Richardson, G. Allen, and **P. Monahan**. Thromboembolism in Childhood. Current Perspective and Distinct Challenges. *Thrombosis and Haemostasis*, 88(6):900-911, 2002.

D. M. McCarty, H. Fu, **P. E. Monahan**, C. E. Toulson, and R. J. Samulski. Adeno-associated Virus Terminal Repeat (TR) Mutant Generates Double-strand Vectors to Overcome Rate-limiting Step in Transduction in Vivo. *Gene Therapy*, 10:2112-2118, 2003.

M. G. Douvas and **P. E. Monahan**. Life-threatening Thrombosis Complicating the Management of Hepatic Hemorrhage: Anticoagulant Treatment in a Newborn with Hemophilia B. *Journal of Pediatric Hematology/Oncology*, 2004 (In press).

C. RESEARCH SUPPORT

Ongoing Research Support

P01-HL66973 09/01/2000 – 07/31/2004
Factor IX Mouse Models for Hemophilia B Gene Therapy
NIH/NHLBI
Overall project goal: This work generates several strains of mice expressing human factor IX molecules, for investigation of the role of human factor IX/collagen interaction in hemostasis and to serve as models for factor IX gene therapy safety and efficacy studies.
Role: Co-Investigator

07/03-(renewable thru 2006)
Double-stranded Adeno-associated Virus (AAV) Vectors to Improve Efficacy and to Evaluate Safety of Factor IX Gene Therapy
National Hemophilia Foundation, Career Development Award
Overall project goal: This study investigates the potential of double-stranded AAV vectors to achieve tolerance of factor IX and therapeutic expression in the context of liver-targeted gene therapy.
Role: PI

07/03-(renewable thru 2008)
Overcoming Limited Clotting Factor Gene Transfer to the Liver Using Adeno-associated Virus (AAV) Vectors Presenting a More Active Molecular Form
Hemophilia of Georgia Clinical Scientist Development Grant
Overall project goal: This study investigates the use of self-complementary vector sequences to result in early and widespread liver transduction in available animal models of hemophilia B.
Role: PI

Completed Research Support (Recent)
K08 HL03960 02/99-11/03
AAV-Directed Muscle Gene Therapy for Hemophilia B
NIH/NHLBI Mentored Clinical Scientist Development Award
Overall project goal: This study investigated AAV-directed expression of factor IX in the muscle, examining rate-limiting factors in the kinetics of expression of factor IX from this tissue.
Role: PI (Sponsor: R. Jude Samulski, PhD. Co-sponsor: Gilbert White, II, MD)

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Timothy C. Nichols, M.D.	Professor of Medicine, Pathology and Lab Medicine

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Stanford University, Stanford, CA	B.S.	1974	Biology
Medical College of Virginia, Richmond, VA	M.D.	1978	Medicine
North Carolina Memorial Hospital	Intern, Resident, Chief Resident	1978-1982	Internal Medicine
North Carolina Memorial Hospital	Fellow	1982-1985	Cardiology

A. POSITIONS AND HONORS

Positions and Employment

1981-1985 Clinical Instructor, Department of Internal Medicine, North Carolina Memorial Hospital
1985-1986 Instructor in Medicine, Department of Internal Medicine, North Carolina Memorial Hospital
1986-1993 Assistant Professor of Medicine, North Carolina Memorial Hospital
1991-1993 Assistant Professor of Pathology, University of North Carolina, Chapel Hill
1993-1999 Associate Professor of Medicine and Pathology, University of North Carolina, Chapel Hill
1999-present Professor of Medicine and Pathology, University of North Carolina, Chapel Hill

Other Experience and Professional Memberships (in past 3 years)

NIH Study Section: NHLBI Program Project Grant Reviewer 2003

Honors

1974 Graduated with Department Honors in Biology, Stanford University
1975 Summer Research Award, Richmond Chapter, American Psoriasis Association
1982 NRSA Training Grant in Experimental Cardiology
1987 Recipient of Young Investigator Award, International Society for Thrombosis and Hemostasis, XI Congress, Brussels
1988 James W. Woods Faculty Award
1992-1993 Kenan Fellowship

B. SELECTED PEER-REVIEWED PUBLICATIONS (in chronological order, selected from 52)

T. C. Nichols, J. Roussi, D. A. Bellinger, C. M. Samama, R. L. Reddick, M. Bonneau, M. S. Read, O. Bailliart, G. G. Koch, M. Vaiman, J. L. Sigman, G. A. Pignaud, K. M. Brinkhous, T. R. Griggs and L. Drouet. Function of von Willebrand Factor After Crossed Bone Marrow Transplantation Between Normal and von Willebrand Disease Pigs: Effect on Arterial Thrombosis in Chimeras, *Proc. Natl. Acad. Sci. USA*, 92:2455-2459, 1995.

M. S. Read, R. L. Reddick, A. P. Bode, D. A. Bellinger, **T. C. Nichols**, K. Taylor, S. V. Smith, D. K. McMahon, T. R. Griggs and K. M. Brinkhous. Preservation of Hemostatic and Structural Properties of Rehydrated Lyophilized Platelets: Potential for Long-term Storage of Dried Platelets for Transfusion, *Proc. Natl. Acad. Sci. USA,* 92:391-401, 1995.

P. E. Monahan, J. Tazelaar, **T. C. Nichols**, D. A. Bellinger, M. S. Read, C. E. Walsh and R. J. Samulski. Sustained Expression of Human Factor IX in a Hemophilia B Canine Following Intramuscular Recombinant AAV Gene Transfer, *Human Gene Therapy*, 5:40-49 1998.

H. Chao, R. Samulski, D. Bellinger, P. Monahan, **T. C. Nichols** and C. Walsh. Persistent Expression of Canine Factor IX in Hemophilia B Canines, *Gene Ther*, 6:1695-704, 1999.

R. W. Herzog, E. Y. Yang, L. B. Couto, J. N. Hagstrom, D. Elwell, P. A. Fields, M. Burton, D. A. Bellinger, M. S. Read, K. M. Brinkhous, G. M. Podsakoff, **T. C. Nichols**, G. J. Kurtzman and K. A. High, Long-term Correction

of Canine Hemophilia B by Gene Transfer of Blood Coagulation Factor IX Mediated by Adeno-Associated Viral Vector, *Nature Medicine*, 5:56-63, 1999.

R. O. Snyder, C. Miao, L. Meuse, B. A. Donahue, H-F Lin, D. W. Stafford, S. Patel, A. Thompson, **T. C. Nichols**, D. Bellinger, M. Read, K. M. Brinkhous and M. A. Kay. Correction of the Bleeding Disorder in Canine and Murine Models of Hemophilia B Using Recombinant Adeno-Associated Viral Vectors, *Nature Medicine*, 5:64-70, 1999.

R. Herzog, V.R. Arruda, T.H. Fischer, M.S. Read, **T.C. Nichols** and K.A. High. Absence of Circulating Factor IX Antigen in Hemophilia B Dogs of the UNC-Chapel Hill Colony, *Thromb Haemost*, 84:352-354, 2000.

L. Wang, **T. C. Nichols**, M .S. Read, D. A. Bellinger and I. M. Verma. Sustained Expression of Therapeutic Level of Factor IX in Hemohilia B Dogs by AAV-Mediated Gene Therapy in Liver, *Molecular Therapy*, 1:154-158, 2000.

V. R. Arruda, P. A. Fields, R. Milner, L. Wainwright, P. De Miguel, P. J. Donavan, R. W. Herzog, **T. C. Nichols**, J. A. Biegel, M. Razavi, M. Dake, D. Huff, A. W. Flake, L. Couto, M. Kay and K. A. High. Lack of Germline Transmission of Vector Sequences Following Systemic Administration of Recombinant AAV-2 Vectors in Males, *Molecular Therapy*, 4:586-592, 2001.

K. E. Russell, M. S. Read, D. A. Bellinger, K. Leitermann, B. J. Rup, K. P. McCarthy, J. C. Keith, R. G. Schaub, S. P. Khor and **T. C. Nichols**. Intratracheal Administration of Recombinant Human Factor IX (Benefixtm) Achieves Therapeutic Levels in Hemophilia B Dogs, *Thromb Haemost*, 85:445-9, 2001.

K. M. Brinkhous, H. Sandberg, L. Widlund, M. Read, **T. Nichols**, J. Sigman, U. Oswaldsson, R. G. Schaub and M. Mikaelsson. Preclinical Pharmacology of Albumin-Free B-Domain Deleted Recombinant Factor VIII, *Seminars in Thromb and Haemost*, 28:269-272, 2002.

R. W. Herzog, P. A. Fields, V. R. Arruda, J. Brewbaker, E. Armstrong, D. McClintock, D. A. Bellinger, L. B. Couto, **T. C. Nichols** and K. A. High. Influence of Vector Dose on Factor IX-Specific T and B Cell Responses in Muscle-Directed Gene Therapy for Canine Hemophilia B, *Human Gene Therapy*, 13:1281-1291, 2002.

J. N. Lozier, A. Dutra, E. Pak, N. Zhou, Z. Zheng, **T. C. Nichols**, D. A. Bellinger, M. Read and R. A. Morgan. The Chapel Hill Hemophilia: A Dog Colony Exhibits a Factor VIII Gene Inversion, *Proc Natl Acad Sci*, 99:12991-12996, 2002.

J. D. Mount, R. W. Herzog, D. M. Tillson, S. A. Goodman, N. Robinson, M. L. McCleland, D. A. Bellinger, **T. C. Nichols**, V. R. Arruda, C. D. Lothrop Jr. and K .A. High. Sustained Phenotypic Correction of Hemophilia B Dogs with a Factor IX Null Mutation by Liver-Directed Gene Therapy, *Blood*, 99:2670-2676, 2002.

E. H. N. Olsen, A. S. McCain, E. P. Merricks, T. H. Fischer, I. M. Dillon, R. A. Raymer, D. A. Bellinger, S. A. Fahs, R. R. Montgomery, J. C. Keith Jr, R. G. Schaub and **T. C. Nichols**. Comparative Response of Plasma VWF in Dogs to Up-Regulation of VWF Mrna by Interleukin-11 Versus Weibel-Palade Body Release by Desmopressin (DDAVP), *Blood*, 102:436-441, 2003.

L. Xu, C. Gao, M. S. Sands, S. R. Cai, **T. C. Nichols**, D. A. Bellinger, R. A. Raymer, S. McCorquodale and K. P. Ponder. Neonatal or Hepatocyte Growth Factor-Potentiated Adult Gene Therapy with a Retroviral Vector Results in Therapeutic Levels of Canine Factor IX for Hemophilia B, *Blood*, 101:3924-3932, 2003.

A. Ehrhardt, H. Xu, A. M. Dillow, D. A. Bellinger, **T. C. Nichols** and M. A. Kay. A Gene Deleted Adenoviral Vector Results in Phenotypic Correction of Canine Hemophilia B without Liver Toxicity or Thrombocytopenia, *Blood*, 102:2403-11, 2003.

K. E. Russell, E. H. N. Olsen, R. A. Raymer, E. P. Merricks, D. A. Bellinger, M. S. Read, B. J. Rup, J. C. Keith Jr, K. P. McCarthy, R. G. Schaub and **T. C. Nichols**. Reduced Bleeding Events with Subcutaneous Administration of Recombinant Human Factor IX (Benefixtm) in Immune Tolerant Hemophilia B Dogs, *Blood*, 102:4393-4398, 2003

C. RESEARCH SUPPORT

<u>Ongoing Research Support</u>

2 R24-HL63098 05 (Nichols TC) 07/01/03-06/30/08
NIH/NHLBI
Maintenance of Animal Models of Human Hemophilia and vWD
Overall project goal: Production and maintenance of the hemophilic and von Willebrand disease animals for performing collaborative research studies.
Role: PI

R01 HL69364 (Clemmons DR) 09/30/01-08/31/06
NIH/NHLBI
Atherosclerosis in Insulin-Resistant, Hyperlipidemic Pigs

Overall project goal: Development and characterization of insulin resistant atherosclerotic pigs.
Role: Co-PI

P01 HL66973-01A1 (Samulski RJ) 09/30/01-07/31/06
NIH/NHLBL
Gene Therapy Pulmonary & Hematologic Disorders
Overall project goal: Studies to optimize gene therapy of hemophilia.
Role: Co-Investigator on sub-project.

R01 (Ponder K, Washington Univ) 02/01/02 – 01/31/06
NIH/NHLBI
Gene Therapy Blood Protein Deficiencies
Overall project goal: Studies of gene therapy for blood protein deficiencies.
Role: PI on subcontract at UNC

PPG (Steer C, Univ of Minnesota) 09/14/01-06/30/05
NIH/NHLBI
Chimeroplasty for Factor IX and VIII Gene Expression
Overall project goal: Studies of chimeroplasty for Factor IX and VIII gene expression.
Role: PI for subcontract at UNC

P01-HL64190 (High K, Univ. of Penn.) (Nichols, T.C.) 12/01/99-11/30/04
NIH/NHLBI
Gene Therapy for Hemophilia
Overall project goal: Studies to optimize gene therapy of hemophilia utilizing muscle and liver as target organs; emphasis is placed on the immune response of the host.
Role: PI on subcontract at UNC

P-60-DE13079 (Flood P) 08/01/99-07/30/04
NIH/NIDCR
Activation of NF-kappa B and Oral Inflammation in Atherogenesis
Overall project goal: Studies focused on activation of NF-kappa B and oral inflammation in atherogenesis.
Role: PI for Project 4

U01DE13940 (Offenbacher S) 07/15/01-07/14/04
NIH
A Periodontal Program to Prevent Cardiovascular Events
Overall project goal: A pilot clinical trial to determine the role of periodontitis on cardiovascular events.
Role: Co –PI

<u>Completed Research Support (past 3 years)</u>

1R01 HL52706-01A2 (Lord, Susan) 04/01/98 - 03/31/03
NIH/NHLBI
Fibrinogen As A Determinant In Cardiovascular Disease
Overall project goal: Studies focused on the role of fibrinogen in atherogenesis using a newly created hyperfibrinogenemic mouse.
Role: Co-Investigator

R01-HL61921 (Katherine High, Univ. of Penn) (Nichols, T.C.) 09/30/98 – 09/29/02
NIH/NHLBI
Inhibitor Formation in Gene Therapy for Hemophilia
Overall project goal: Studies on the mechanisms of inhibitor formation during gene therapy for hemophilia.
Role: PI on subcontract at UNC and director of gene therapy studies in hemophilic dogs.

P50-HL54500 (Bennett/ High, Univ. of Penn.)(Nichols, T.C.) 06/01/97–01/31/02
NIH/NHLBI
Intramuscular Injection of AAV-F.IX to Treat Canine Hemophilia B

Overall project goal: Studies to optimize safety and efficacy of skeletal muscle based gene therapy for hemophilia
Role: PI on subcontract at UNC and director of gene therapy studies in hemophilic dogs

ONR N00014-97-1-0891 (Read, M.S.) 06/14/97 - 05/31/01
Office of Naval Research
Studies to Evaluate Efficacy and Safety of Infusable Rehydrated Platelets in Animal Models
Overall project goal: Studies to evaluate efficacy and safety of infusable rehydrated platelets.
Role: Co-Investigator

ONR N00014-97-1-0867 (Read, M.S.) 06/14/97 - 04/30/01
Office of Naval Research
Lyophilized Red Blood Cells and Platelets: Preservation of Oxygen Delivery Capabilities of RBCs and Hemostatic Properties of Platelets
Overall project goal: Studies of preservation of oxygen delivery capabilities of RBCs and hemostatic properties of rehydrated platelets.
Role: Co-Investigator

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
S. Todd Swanson, Ph.D.	Research Assistant Professor Department of Chemical Engineering

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Lewis and Clark College, Portland, OR	B.S. (w/honors)	1985	Chemistry
University of California, San Diego	M.S.	1992	Chemistry
University of California, San Diego	Ph.D.	1993	Chemistry

A. POSITIONS AND HONORS

Positions and Employment

1986-1987 Quality Control Chemist, Hall Laboratories, Portland, Oregon
1988-1992 Teaching Assistant, Dept of Chemistry, University of California, San Diego
1996 Instructor, Dept of Biochemistry, University of Nebraska-Lincoln
1993-1997 Post-doctoral Researcher, University of Nebraska-Lincoln
1997-2000 Chilton Foundation Fellow, U.T. Southwestern Medical Center at Dallas (TX)
2000-2002 Supervisor, Analytical Development Laboratory, University of Nebraska-Lincoln
2002-present Research Assistant Professor, Dept of Chemical Engineering, University of Nebraska-Lincoln

B. SELECTED PEER-REVIEWED PUBLICATIONS (in chronological order)

S. T. Swanson and D. Roise. Protein Import into Mitochondria. *Biophysical Journal*, 57(2):A198, part 2, 1990.

S. T. Swanson and D. Roise. Binding of a Mitochondrial Presequence to Natural and Artificial Membranes: Role of the Surface Potential. *Biochemistry,* 31:5746-5751, 1992.

S. T. Swanson, H. Fang, and M. H. O'Leary. Are All Ribulose 1,5-bisphosphate carboxylase/oxygenases the Same?" *FASEB Journal*, 9(6):A1481, 1995.

S. Madhavan, **S. T. Swanson**, T. M. Blackmer, T. S. Pinkerton, and M. H. O'Leary, M. H. Relationship between Nitrogen Nutrition and Photosynthetic Carbon Isotope Fractionation in Corn. *Plant Physiology*, 111(2):336, Supplement S, 1996.

T. Swanson, H.B. Brooks, A. L. Osterman, M. H. O'Leary, and M. A. Phillips. Carbon-13 Isotope Effect Studies of *Trypanosoma brucei* Ornithine Decarboxylase. *Biochemistry*, 37:14943-14947, 1998.

S. T. Swanson, D. W. Foster, J. D. McGarry, and N. F. Brown. Roles of the N- and C-terminal Domains of Carnitine Palmitoyltransferase I Isoforms in Malonyl-CoA Sensitivity of the Enzymes: Insights from Expression of Chimaeric Proteins and Mutation of Conserved Histidine Residues. *Biochemical Journal*, 335:513-519, 1998.

S. T. Swanson, J. Rudzinski, P. Paneth, and M. H. O'Leary. Carbon and Chlorine Kinetic Isotope Effects and Solvent Effects on the Hydrolysis of Chloroformates. *Polish Journal of Chemistry*, 76:1721–1731, 2002.

J. J. Robinson, K. M. Scott, **S. T. Swanson**, M. H. O'Leary, K. Horken, F. R. Tabita, and C. M. Cavanaugh. Kinetic Isotope Effect and Characterization of Form II RubisCO from the Chemoautotrophic Endosymbionts of the Hydrothermal Vent Tubeworm, *Riftia pachyptila*. *Limnology and Oceanography* 48(1):48-54, 2003.

S. K. Johnson, W. Zhang, L. A. Smith, K. J. Hywood-Potter, **S. T. Swanson**, V. L. Schlegel, and M. M. Meagher. Scale up of the Fermentation and Purification of the Recombinant Heavy Chain C of Botulism nNurotoxin Serotype F, Expressed in *Pichia pastoris. Protein Expression and Purification*, 32(1):1-9, 2003.

C. RESEARCH SUPPORT

Ongoing Research Support

U01-AI056514-01 09/02/03 – 09/01/07
NIH
Fast Track Development of a Heptavalent Vaccine Against the Botulinum Neurotoxin
Overall project goal: The goal of this project is to complete all aspects of process development for a recombinant vaccine against the botulinum neurotoxin. This includes molecular biology, fermentation, purification, analytical methods development, and technology transfer for cGMP production of serotypes C, D, E, F, and G of the botulinum neurotoxin.
Role: Co-PI: Manage Quality Control Laboratory and manage Analytical Methods Laboratory.

DAMD17-02-1-0659 03/15/02-03/14/05
UAMRMC
Process Research and Development of Antibodies as Countermeasures for *C. botulinum*
Overall Project Goal: The goal of this project is to express and optimize expression in CHO cells of human antibodies against the the botulinum neurotoxin. The goal of the this project is to fill out laboratory space on third floor of Othmer Hall, new home for the UNL Biological Process Development Facility
Role: Key Person: Provide analytical and quality control support for cell culture experiments.

DAMD17-02-C-0107 01/01/02 – 01/01/05
USAMRMC
Process Research and Development for Therapeutic Agents and Vaccines as Countermeasures Against Biological Warfare Agents
Overall project goal: The goal of this project is the research and development of processes to produce vaccines and therapeutic agents against biological threats. The Army needs countermeasures to protect combat and non-combat personnel against biological threats.
Role: Key Person: Provide analytical methods development and quality control for process development.

Completed Research Support

DAMD17-98-C8034 01/01/98 – 12/30/01
USAMRMC
Fermentation Process Development for the Production of the Hc Fragment of the Botulinum Neurotoxin Serotype A
Overall project goal: This is part of the project “Fermentation, Recovery, and Purification of the Hc Fragment of the Botulinum Neurotoxin from *Pichia pastoris*” contracted with USAMRMC. The goal is to develop a workable and scalable fermentation and purification process research and development of botulinum vaccine candidates.
Role: Key Person: Quality control and analysis of in-process samples.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Kevin E. Van Cott, Ph.D.	Assistant Professor, Department of Chemical Engineering

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Purdue University	B.S.	1991	Chemical Engineering
Virginia Technological Institute	Ph.D.	1996	Chemical Engineering
Virginia Technological Institute	Post-doc	1996	Chemical Engineering

A. POSITIONS AND HONORS

Positions and Employment

1991 Exxon Production Research, Houston, TX (engineering internship)
1996-1998 Research Scientist, F&S Inc., Blacksburg, VA (now Luna Innovations Inc.)
1998-present Assistant Professor, Virginia Technological Institute

Other Experience and Professional Memberships

ProGenetics LLC (Blacksburg, VA) Animal Care Committee; Founding member of the Center for Self-Assembled Nanostructures and Devices at Virginia Tech; Faculty Advisor: Omega Chi Epsilon (Chemical Engineering Honor Society); Undergraduate Curriculum Committee, Department of Chemical Engineering; Referee: *Journal of Dairy Science, Biotechnology Progress, Sensors and Materials, Biotechnology and Bioengineering*

Honors

1996 Paul Torgersen Excellence in Research Award – 1st Place for best Ph.D. Dissertation in the College of Engineering at Virginia Tech

B. SELECTED PEER-REVIEWED PUBLICATIONS (in chronological order)

K. E. **Van Cott**, R. D. Whitley, N. H. L. Wang. Effects of Temperature and Flow Rate on Frontal and Elution Chromatography of Aggregating Systems. *Separations Technology*, 1**:**142-152, 1991.

R. D. Whitley, **K. E. Van Cott**, J. A. Berninger, N. H. L. Wang. Effects of Protein Aggregation in Isocratic Nonlinear Chromatography. *AIChE J.,* 37(4):555-568, 1991.

R. D. Whitley, **K. E. Van Cott**, N. H. L. Wang. Analysis of Non-equilibrium Adsorption/Desorption Kinetics and Implications for Analytical and Preparative Chromatography. *Ind. Eng. Chem. Res.*, 32:149-159, 1993.

A. Subramanian, **K. E. Van Cott**, D. S. Milbrath, W. H. Velander. Role of Local Antibody Density Effects on Immunosorbent Efficiency. *Journal of Chromatography*, 672:11-24, 1994.

S. P. Butler, **K. Van Cott**, A. Subramanian, F. C. Gwazdauskas, W. H. Velander. Current Progress in the Production of Recombinant Human Fibrinogen in the Milk of Transgenic Animals. *Thrombosis and Haemostasis*, 78(1):537-542, 1997.

K. E. Van Cott, H. Lubon, C. G. Russell, S. P. Butler, F. C. Gwazdauskas, J. Knight, W. N. Drohan, W. H. Velander. Phenotypic and Genotypic Stability of Multiple Lines of Transgenic Pigs Expressing of Recombinant Human Protein C. *Transgenic Research*, 6:203-212, 1997.

K. E. Van Cott, B. L. Williams, F. C. Gwazdauskas, H. Lubon, T. Lee, W. N. Drohan, W. H. Velander. Affinity Purification of Biologically Active and Inactive Forms of Recombinant Human Protein C Produced in the Porcine Mammary Gland. *Journal of Molecular Recognition*, 9:407-414, 1997.

K. Van Cott and W. H. Velander. Transgenic Animals as Drug Factories: A New Source of Recombinant Protein Therapeutics. *Expert Opinion on Investigational Drugs*, 7(10):1683-1690, 1998.

K. Van Cott, S. P. Butler, C. G. Russell, A. Subramanian, H. Lubon, F. C. Gwazdauskas, J. Knight, W. N. Drohan, W. H. Velander. Transgenic Pigs as Bioreactors: A Comparison of Gamma-Carboxylation of Glutamic Acid in Recombinant Human Protein C and Factor IX by the Mammary Gland. *Genet Anal* 15(3-5):155-60, 1999.

C. Brands, P. Neyman, M. T. Guzy, S. Shah, H. W. Gibson, **K. E. Van Cott**, R. M. Davis, J. R. Heflin Jr. In Situ Second Harmonic Generation Measurements of the Growth of Nonlinear Optical Ionically Self-Assembled Monolayers. *Polym. Mater. Sci. Eng.* (Am. Chem. Soc. Div. Polym. Mater. Sci. Eng.), 83:219-220, 2000.

M. T. Guzy, S. Shah, R. M. Davis, **K. E. Van Cott**, J. R. Heflin, H. Wang, H. W. Gibson. Novel Deposition Techniques for Self-Assembled Non-Linear Optic Thin Films. *Polym. Mater. Sci. Eng.* (Am. Chem. Soc. Div. Polym. Mater. Sci. Eng.) 83:260, 2000.

P. J. Neyman, M. T. Guzy, S. Shah, H. Wang, H. W. Gibson, **K. E. Van Cott**, R. M. Davis, J. R. Heflin. Second Order Nonlinear Optical Properties of Ionically Self-Assembled Films Containing Dianionic Chromophores. *Polym. Mater. Sci. Eng.* (Am. Chem. Soc. Div. Polym. Mater. Sci. Eng.), 83:162-163, 2000.

K. E. Van Cott, H. Lubon, F. C. Gwazdauskas, J. Knight, W. N. Drohan, W. H. Velander. Recombinant Human Protein C Expression in the Milk of Transgenic Pigs and the Effect on Endogenous Milk Immunoglobulin and Transferrin Levels. *Transgenic Research*, 10:43-51, 2001.

K. E. Van Cott, M. Guzy, P. Neyman, C. Brands, J. R. Helfin, H. W. Gibson, R. M. Davis. Layer-by-Layer Deposition and Ordering of Low-molecular Weight Dye Molecules for Second-order Nonlinear Optics. *Angew. Chem. Int. Ed.* 41(17):3236-33238, 2002.

K. E. Van Cott, M. Guzy, R. M. Davis, H. W. Gibson, J. R. Heflin. Characterization of the Purity and Stability of Commercially Available Dichlorotriazine Chromophores Used in Nonlinear Optical Materials. *Dyes and Pigments*, 58(2):145-155, 2003.

W. H. Velander and **K. E. Van Cott**. Protein Expression Using Transgenic Animals. *Handbook of Industrial Cell Culture*, ed. Vinci VA, Parekh SR. Humana Press, 51-67, 2003.

D. Balasubramanian, C. Wilkinson, **K. Van Cott**, C. Zhang. Tobacco Protein Separation by Aqueous Two-phase Extraction. *Journal of Chromatography*, 989(1):119-29, 2003.

M. Lindsay, G. Gil, C. Zhang, A. Cadiz, W. H. Velander, **K. E. Van Cott**. Purification of Recombinant DNA-derived Factor IX and Fractionation of Active and Inactive Subpopulations. *Journal of Chromatography*, 1026:149-157, 2004.

M. Guzy, R. M. Davis, P. Neyman, C. Brands, J. R. Heflin, H. W. Gibson, **K. E. Van Cott**. Self-assembled Organic Films: Covalent/ionic Self-assembly for Second-order Nonlinear Optical Materials. *Encyclopedia of Nanotechnology*, 2004 (In press).

C. RESEARCH SUPPORT

Ongoing Research Support

ARM-2T-1026/488-VT 08/18/03-08/17/05
DOD-STTR Phase II
Fluorescent, Polymerized, Affinity Liposomes for the Detection of Bacterial Toxins
Overall project goal: Produce, purify, and characterize stable liposomes that can be used for new specific and sensitive assays for bacterial toxins (cholera toxin, anthrax lethal factor).
Role: PI

ARM-2T-1027/489-VT 08/18/03-08/17/05
DOD-STTR Phase II
Viability Assays for Monitoring Decontamination of Pathogenic Bacteria
Overall project goal: Determine how new decontamination agents the U.S. Army is developing affects bacterial toxin structure and function.
Role: PI

Completed Research Support

ARM-1S-1026/286-VT 09/15/02- 01/15/03
DOD-STTR Phase I
Fluorescent, Polymerized, Affinity Liposomes for the Detection of Bacterial Toxins
Overall project goal: Produce, purify, and characterize stable liposomes that can be used for new specific and sensitive assays for bacterial toxins (cholera toxin, anthrax lethal factor).
Role: PI

BES-0086876 09/1/00-08/31/02
NSF
Monodisperse Block Copolymers for Environmentally-Friendly Processing of Aqueous Metal Oxide Suspensions
Overall project goal: Develop new cloning strategies to produce synthetic protein polymers.
Role: Co-PI

NSF-2T-7002 11/15/01-07/15/02
NSF-STTR Phase I
Optic Fiber Sensors for the Detection of Pathogenic Microorganisms
Overall project goal: Determine if Long Period Grating fiber optics can be used to detect the presence of pathogenic bacteria or bacterial toxins.
Role: PI

BES-0103037 06/15/01-05/31/02
NSF
Detecting Specific Biomolecule Adsoprtion in Ionically Self-assembled Monolayers via Second Harmonic Generation
Overall project goal: Exploratory grant to see if second harmonic generation can be used to specifically detect the adsorption of a biomolecule (protein or DNA) to a surface.
Role: Co-PI

ECS-9907747 09/01/99-08/31/01
NSF
Supramolecular Self-assembly of Second Order Nonlinear Optical Materials for Electro-optic Devices
Overall project goal: Design new materials and strategies for making nonlinear optical materials (materials that have a high degree of polar ordering of the constituents.
Role: Co-PI

NSF (REU Supplement) 07/15/00-03/15/01
Supramolecular Self-Assembly of Second Order Nonlinear Optical Materials for Electro-optic Devices
Overall project goal: Undergraduate research project.
Role: Co-PI

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

William H, Velander, Ph.D., Principal Investigator – University of Nebraska-Lincoln

Laboratory:
Not Applicable

Clinical:
Not Applicable

Animal:
Not Applicable

Computer:
Dr. Velander has a Sony Viao 1.7 GHz laptop computer with full access to the internet, network color laser printer, and the Biological Process Development Facility (BPDF) local area network with access to the Storage Area Network.

Office:
Dr. Velander has a separate, private office, approximately 160 square feet with full access to the Department of Chemical Engineering office equipment, fax and copier. In addition, Dr. Velander has full access to the BPDF's computer support staff and systems.

Other:
Access to fax and scanners for document processing.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Not Applicable

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Analytical Methods Laboratory – University of Nebraska-Lincoln

Laboratory:

The Analytical Method Development Laboratory is co-located with the Quality Control Laboratory in a combined biochemistry and chemistry laboratory with approximately 900 square feet of space is located in Room 310. The room is equipped with an eight-foot chemical fume hood and secured cold storage for short-term samples. In the service corridor is secured long-term cold storage for 4 °C, -20 °C, and –80 °C samples.

Clinical:

Not Applicable

Animal:

Not Applicable

Computer:

The laboratory is equipped with several computer systems as listed in the major equipment section below. The minimum instrument workstation is a Pentium II with 128 Mb of Ram, a 20 Gb hard drive, NIC, and color monitor running a Windows 2000 workstation. Each laboratory has at least two computer workstations that are similarly equipped. Of major note is the client/server system for high performance liquid chromatography instrument control, data acquisition, data analysis, and data reporting. This system consists of two Waters Lac/e32 Acquisition servers (Pentium III with 512 Mb Ram and 40 Gb running Windows 2000 workstation SP4 operating system) and a Dell PowerEdge 2500 server (1.3 GHz Pentium III, 1 Gb RAM, 4 x 36 Gb Hard drives set up in two raid 1 (mirrored) arrays, one for operating and programs system and another for the Empower Oracle database) running a Windows 2000 server SP4 operating system. The clients, acquisition servers, and the database server run Waters Empower software, version 5.0. The client/server system is accessed by software clients, which are installed on several workstations in the laboratory and the computers in Dr. Swanson's and Analytical Methods laboratory personnel offices. The current office computer in service is a Latitude 840C laptop, 2.2 GHz Pentium III, 512 Mb RAM, 40 Gb hard drive, CD-R/RW/DVD, interchangeable 250 Mb ZIP drive or 1.44 Mb floppy disk drive, two batteries (six hours time on batteries), fax/modem, Ethernet NIC, two USB ports, external mouse, and keyboard or equivalent. The computers are pre-loaded with Microsoft Office suite, Waters Millennium 32 software, and ancillary software programs. All computers are interconnected via the University of Nebraska LAN system and protected from external tampering by a Biological Process Development Facility (BPDF) controlled firewall.

Office:

A separate, private office, approximately 120 square feet, is available to Dr. Todd Swanson, Manager of both the Analytical Methods Laboratory and the Quality Control Laboratory. The technologists will each have a desk area. Technicians are provided locker storage for personal items.

Other:

The Analytical Methods Development Laboratory will request amino acid analysis and amino terminal amino acid sequencing from two contract facilities: 1) The Protein Structure Core Facility of the University of Nebraska Medical Center in Omaha is equipped with and is capable of performing cGLP amino terminal amino acid sequencing. The equipment includes an Applied Biosystems Procise protein sequencer and an Applied Biosystems 477 protein sequencer. 2) AAA Service Laboratory, Boring, OR, uses a Beckman 6300 Amino Acid Analyzer for performing cGLP compliant amino acid analysis. We have existing working relationships with both facilities.

Mass Spectrometry services will be acquired on a fee-for-service basis from contract service laboratories depending on our needs. Locally, the University of Nebraska-Lincoln's Nebraska Center for Mass Spectrometry offers some of the services needed. The University of California, Davis' Molecular Structure Facility offers an extensive list of mass spectrometry services at attractive non-profit rates.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

- 3 HPLC systems:
  1) Waters HPLC 2695 Alliance System with a 2996 photodiode array absorbance detector and a 2420 evaporative light scattering detector; 2) Waters 600 Modular HPLC System with a 717+ autosampler and a 2487 dual wavelength absorbance detector; and 3) Waters 600 Modular HPLC System with manual injector and a 2487 dual wavelength absorbance detector. System control, data acquisition, data analysis, and data reporting are accomplished by using a Waters Lac/E32 acquisition server and a Database server both running the Empower software package for HPLC systems (see computer section above).The database server with Empower software package for HPLC systems is operated by the Quality Control Laboratory but shared with the Analytical Methods Laboratory (see computer section above). *Capabilities*: 1) Solvent delivery – modular systems can deliver flow rates for analytical and semi-preparative HPLC columns; Alliance system can deliver flow rates for capillary to semi-preparative columns. All systems are capable of generating quaternary gradients with a 1% composition accuracy. 2) Detection – all absorbance detectors have wavelength range of 190 nm to 800 nm and are equipped with 10 microliter analytical flow cell with a 1 cm path length. Data can be acquired up to a rate of 10 per second for a single wavelength and 1 per second for dual wavelength acquisition (10 spectra per second for the photodiode array detector). 3) Autosamplers – both 48 and 96 sample carousels are available for the 717+ autosamplers. The 2695 Alliance systems have five carousel, turntable autosamplers built-in with the capacity for 120 vials. Sample compartments are temperature controlled 0°C to 40°C. Currently all these systems are validated.
- Beckman MDQ capillary electrophoresis system with a filter-based UV-visible absorbance detector and a photodiode array detector (attached to a computer) instrument operation, data acquisition, and data analysis are controlled by 32 Karat software. *Capabilities*: 96 well plates with robotics system; internal liquid cooling system for the capillary, 30,000 kV maximum voltage, ability to perform capillary zone electrophoresis, capillary gel electrophoresis, capillary isoelectric focusing, and capillary electrochromatography. This system can be validated.
- Dynex MRX filter based, UV-visible absorbance microplate reader and computer software for data acquisition and analysis. *Capabilities*: multiwell plates up to 96 wells; equipped with filters for 215 nm, 405 nm, 450 nm, and 590 nm.
- Multiwell plate washer system
- The equipment of the Quality Control Laboratory will be accessible to the Analytical Methods Laboratory on a need and availability basis. This is a reciprocal agreement requiring both parties to adhere to the highest quality standards required, e.g., cGLP or cGMP.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Analytical Methods and Characterization, Kevin E. Van Cott, Ph.D. – University of Nebraska-Lincoln

Laboratory:

Dr. Van Cott will be moving to UNL in May 2004. He will have 700 sq ft of laboratory space in 330 Othmer Hall, adjacent to the Biological Process Development Facility (BPDF) laboratories. His laboratory at UNL will be similar to what he currently has at Virginia Tech (as of January 2004).

Clinical:

Not applicable

Animal:

Not applicable

Computer:

Dr. Van Cott will have one desktop office computer and one notebook computer. The laboratory will be equipped with dedicated computers and printers for each HPLC, FPLC, and Capillary electrophoresis unit.

Office:

An office space in Othmer Hall has been assigned to Van Cott.

Other:

Dr. Van Cott will have a close association with the UNL BPDF especially the Analytical Methods Laboratory and the Quality Control Laboratory.

Mass Spectrometry services will be acquired on a fee-for-service basis from contract service laboratories depending on our needs. Locally, the University of Nebraska-Lincoln's Nebraska Center for Mass Spectrometry offers some of the services needed. The University of California, Davis' Molecular Structure Facility offers an extensive list of mass spectrometry services at attractive non-profit rates.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following items are in the process of being installed in Van Cott's new laboratory

- Beckman System Gold 32Karat HPLC system with diode array UV/Vis detector and fluorescent detector and fraction collector (330 Othmer): for analysis of rhFIX samples and glycan analysis.
- Beckman System Gold 32Karat HPLC system with diode array UV/Vis detector and fraction collector (330 Othmer): for analysis of samples; high resolution purification.
- Beckman PACE MDQ Glycoprotein Capillary electrophoresis system with UV/Vis and LIF detectors (330 Othmer): for cIEF analysis of rhFIX samples; monosaccharide and oligosaccharide analysis.
- Akta Explorer 100 chromatography system (330 Othmer): chromatography process development; flow rates of up to 100 ml/minute.
- Other related protein characterization equipment in 330 Othmer: Novex SDS PAGE; BioRad Protean IIxi SDS PAGE; Beckman DU-600 UV/Vis spectrophotometer; refrigerated centrifuges (table top and desktop); HPLC columns; lab-scale FPLC columns (up to 5 cm ID); coagulation meter; automated microtiter plate reader; Savant speed-vac system; walk-in cold rooms; -80 freezers; laminar flow hood; fume hood.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Purification – University of Nebraska-Lincoln

Laboratory:

The Purification Development Laboratory is located on the third floor of Othmer Hall on the University of Nebraska-Lincoln campus. The Purification Development Laboratory is a 1,500 square foot newly constructed laboratory with a 150 square foot cold room, dark room (80 square feet), electrophoresis station (SDS-PAGE, IEF, Western blot), and a 200-foot pilot-scale purification area. Also available is a 600 square foot media preparation room. The entire facility and all of its resources will be available for this project.

Clinical:

Not applicable

Animal:

Not applicable

Computer:

Two computer workstations are in the Purification Development Laboratory. Dr. Meagher and the research scientist working on purification have personal computers in their offices.

Office:

Office space is provided on the third floor of Othmer Hall across from the laboratory. The research scientist has an 80 square foot cubical, and Dr. Meagher has a standard single-occupancy office. Research technicians have computer access in the laboratory. There is a locker area for storage of personal items.

Other

There are no other facilities that the purification group will require for this project. If there is a need to fabricate anything (unforeseen at this point), the University of Nebraska-Lincoln Department of Chemistry Machine Shop is available on the first floor of Othmer Hall.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

In the Purification Development Laboratory and under the direct control of the purification group:

2 Perseptive Biosystems Biocad process chromatography workstation and one Perseptive Biosystems Biocad Vision chromatography workstation – attached robot. *Systems have UV, conductivity, and pH monitoring on-line, computer controlled for unattended operation and capable of running multiple columns/buffers systems. Flow rates up to 100 ml/min, and the Vision is equipped with a robot for automated sample analysis;* 1 EH-110 Microfluidizer – *Capable of cell disruption up to 25,000 psig and a flow rate of 400 ml/min*; 1 Amicon K'40 Process chromatography skid – *Capable of gradient, online pH, conducitivity, UV 280, and a flow rate up to 500ml/min;* 1 bench-top ultrafiltration system – *Capable of 8 L/min recirculation rate and a minimum volume of 300 mL and maximum of 3,500 mL. Upper pressure limit of the pump is 100 psig;* 1 NCSRT Model 50 ultrafiltration system – *Capable of 100 L/min recirculation rate and a minimum volume of 3,500 mL. Upper pressure limit of the pump is 100 psig;* 2 floor model Sorval Evolution centrifuges – *Capable of 30,000 x g with a volume capacity of 6 L (8,000 x g);* 1 Beckman J2-21 floor model centrifuge – *Capable of 21,000 x g, upper volume limit 3 L;* 1 Alpha Innotech gel scanning and documentation system – *Capable of complete documentation and scanning including chemillumensence;* 2-by-20 L stainless steel processing tanks – *Capable of mixing and are jacketed.*

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

cGMP Production – University of Nebraska-Lincoln

Process area:

The GMP pilot plants consist of a Fermentation Pilot Plant (FPP) and a Purification Pilot Plant (PPP) and are located on the first floor of Filley Hall on the University of Nebraska-Lincoln campus. The FPP is a 700 square foot room consisting of a 60 square foot centrifuge room. The PPP is a 600 square foot Class 100,000 room with a 220 square foot cold room and a 40 square foot curtained, clean air area containing a clean workstation. Also available is a 150 square foot clean room for inocculum preparation and a 100 square foot chemical weight room. The entire facility and its resources will be available for this project.

Clinical: Not applicable

Animal: Not applicable

Computer:

One computer workstation is located in each of the pilot plants. Managers working on this project have personal computers in their offices, and an operator office is used that contains two computers.

Office:

Office space is provided on the second floor of Filley Hall. A 60 square foot office is used by the GMP pilot plant coordinator, and a 100 square foot office is used by operators of the GMP pilot plants.

Other

The BPDF also has a Quality Control laboratory located on the third floor of Othmer Hall that will perform analysis of cleaning samples and any in-process testing that may be required. If there is a need to fabricate anything (unforeseen at this point), the University of Nebraska-Lincoln Department of Chemistry Machine Shop is available on the first floor of Othmer Hall.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

In the GMP pilot plants and under the direct control of the GMP pilot plant group:

Located in FPP: 1 ABEC 500 L Fermentor. *Capable of high cell density fermentations of Pichia pastoris and Escherichia coli with programmed fixed, ramped or exponential feed profiles. System has control and on-line monitoring of pH, DO, temperature, three different nutrient addition lines, acid/base addition and agitation;* 1 CSC8 Westfalia disk separator. *Capable of 4 lpm flow rates and separating at a maximum of 12 % solid;* 2 NCSRT Model 50 Cross flow filtration systems. *Bleed and fed systems with in-line pH, temperature, conductivity, permeate flow rate monitoring and capable of 800 lpm recirculation rates and operating up to 100 psi;* 1 APV Gaulin 30-3000 Homogenizer. *Capable of cell disruption up to 18,000 psi and a flow rate of 4 lpm;* 1 x 250 L, 1 x 125 L, 1 x 150 L stainless steel mixing/holding tanks. *Jacketed tanks capable of CIP.*

Located in PPP: 1 NCSRT Chromatography skid. *System has in-line UV, pH, temperature, conductivity monitoring with 2 sets of dual metering pumps capable of flow rates ranging from 50 to 700 mL/min and 500 to 7,000 mL/min isocratic. Able to perform linear gradients with five outlets for fractionation if necessary;* 1 NCSRT Model 10 Cross flow filtration skid. *Capable of 80 lpm and a minimum volume of 3 liters with on-line pH, temperature, conductivity and flow rate monitoring;* 1 NCSRT Model 5 Cross flow filtration skid. *Capable of 17 lpm and a minimum volume of 500 mL;.* 1 AirClean Class 100 workstation. *4'x 2'x 3.5' HEPA filtered workstation for use during aseptic manipulation of product and bulking of final product;* 2 x 100/500 BPG column, 2 x 200/500 BPG columns, 1 x 200/1000 BPG column, 1 x 300/500 BPG column, 1 x 450/500 BPG column, 1 x 450/1000 BPG column. *Sanitary borosilicate glass columns commonly used in GMP bioprocessing capable of column volumes ranging from 0.5 to 116 L and pressure limits ranging from 36 to 116 psi.*

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Quality Assurance – University of Nebraska-Lincoln

Laboratory:
Not Applicable

Clinical:
Not Applicable

Animal:
Not Applicable

Computer:
All individuals have Dell Latitude D800 Laptop Pentium M computers with 1.7 Ghz processors, 15.4" WUXGA monitors, 60 GB Hard Drive and 1025 MB RAM. Operating System is Windows XP Professional. Computers are connected to the internet, network color laser printer, and the Biological Process Development Facility (BPDF) local area network with access to the Storage Area Network.

Office:
A separate, private office, approximately 130 square feet, is available to Ms. Ardis Barthuli, Quality Assurance Coordinator for the BPDF. QA Document Specialists are provided a general office area with approximately 90 square feet of office area. There is a separate 1,500 square feet controlled access room with lockable lateral filing cabinets for document storage.

Other:
Access to fax and scanners for document processing.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Not Applicable

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Quality Control – University of Nebraska-Lincoln

Laboratory:

The Quality Control Laboratory is located in several rooms on the third floor of Othmer Hall: Rooms 310, 321, and 334. A combined biochemistry and chemistry laboratory with approximately 900 square feet of space is located in Room 310. The room is equipped with an eight-foot chemical fume hood and secured cold storage for short-term samples. In the service corridor is secured long-term cold storage for 4 °C, -20 °C, and –80 °C samples. A temporary microbiology laboratory consisting of approximately 64 square feet is located in Room 321. This space contains a four-foot biological safety cabinet. The main Quality Control Microbiology Laboratory, Room 334, is currently under design and construction. Current construction time lines suggest that this facility will be built and partially validated by 2005. This facility will consist of two functional spaces: 1) a microbiology laboratory with a class 100,000 environment and 2) a class 10,000 clean room environment with two sequential ante rooms. The class 100,000 laboratory space, approximately 645 square feet, will contain a cGMP autoclave, a four-foot biological safety cabinet, a five-foot biological safety isolator, and a pass-through glove box for transfer of materials from the laboratory to the clean room space. The class 10,000 clean room will be accessed by passage from the hallway into a 120 square foot ante room and gowning area and then into a secondary class 10,000 ante room prior to entry into the clean room. The clean room will contain a 10-foot biological safety isolator. The clean room and all isolators will be sterilized by a hydrogen peroxide vapor generation system.

The Quality Control Laboratories are adjacent to the fermentation, mammalian cell culture, molecular biology, and protein purification laboratories of the Biological Process Development Facility (BPDF). The Nebraska Center for Mass Spectrometry is five minutes walking distance from the laboratory.

Clinical:

Not Applicable

Animal:

Not Applicable

Computer:

The laboratory is equipped with several computer systems as listed in the major equipment section below. The minimum instrument workstation is a Pentium II with 128 Mb of Ram, a 20 Gb hard drive, NIC, and color monitor running Window 2000 workstation. Each laboratory has at least two computer workstations that are similarly equipped. Of major note is the client/server system for high performance liquid chromatography instrument control, data acquisition, data analysis, and data reporting. This system consists of two Waters Lac/e32 Acquisition servers (Pentium III with 512 Mb Ram and 40 Gb running Windows 2000 workstation SP4 operating system) and a Dell PowerEdge 2500 server (1.3 GHz Pentium III, 1 Gb RAM, 4 x 36 Gb Hard drives set up in two raid 1 (mirrored) arrays, one for operating and programs system and another for the Empower Oracle database) running a Windows 2000 server SP4 operating system. The clients, acquisition servers, and the database server run Waters Empower software, version 5.0. The client/server system is accessed by software clients, which are installed on several workstations in the laboratory and the computers in Dr. Swanson's and QC laboratory personnel offices. The current office computer in service is a Latitude 840C laptop, 2.2 GHz Pentium III, 512 Mb RAM, 40 Gb hard drive, CD-R/RW/DVD, interchangeable 250 Mb ZIP drive or 1.44 Mb floppy disk drive, two batteries (six hours time on batteries), fax/modem, Ethernet NIC, two USB ports, external mouse, and keyboard or equivalent. The computers are pre-loaded with Microsoft Office suite, Waters Millennium 32 software, and ancillary software programs. All computers are interconnected via the University of Nebraska LAN system and protected from external tampering by a BPDF controlled firewall.

Office:

A separate, private office, approximately 120 square feet, is available to Dr. Todd Swanson, Manager of both the Analytical Methods Laboratory and the Quality Control Laboratory. The technologists will each have a desk area. Technicians are provided locker storage for personal items.

Other:

The Quality Control Laboratory will request amino acid analysis and amino terminal amino acid sequencing from two contract facilities: 1) The Protein Structure Core Facility of the University of Nebraska Medical Center in Omaha is equipped with and is capable of performing cGLP amino terminal amino acid sequencing. The equipment includes an Applied Biosystems Procise protein sequencer, and an Applied Biosystems 477 protein sequencer. 2) AAA Service Laboratory, Boring, OR, uses a Beckman 6300 Amino Acid Analyzer for performing cGLP compliant amino acid analysis. We have existing working relationships with both facilities.

Mass Spectrometry services will be acquired on a fee-for-service basis from contract service laboratories depending on our needs. Locally, the University of Nebraska-Lincoln's Nebraska Center for Mass Spectrometry offers some of the services needed. The University of California, Davis' Molecular Structure Facility offers an extensive list of mass spectrometry services at attractive non-profit rates.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Biochemistry and Chemistry Laboratory, Othmer 310

- Associates of Cape Cod Pyrokinetics turbidometric system for endotoxin determination (attached to a computer and printer). *Capabilities*: this system has been validated and is capable of simultaneously assaying 96 samples
- Two HPLC systems: 1) Waters HPLC 2695 Alliance System with a 2487 dual wavelength absorbance detector, 2) Waters 600 Modular HPLC System with a 717+ autosampler and a 486 single wavelength absorbance detector. System control, data acquisition, data analysis, and data reporting are accomplished by using a Waters Lac/E32 acquisition server and a Database server both running the Empower software package for HPLC systems (see computer section above). *Capabilities*: 1) Solvent delivery – modular systems can deliver flow rates for analytical and semi-preparative HPLC columns; Alliance system can deliver flow rates for capillary to semi-preparative columns. All systems are capable of generating quaternary gradients with a 1% composition accuracy; 2) Detection – all absorbance detectors have a wavelength range of 190 nm to 800 nm and are equipped with a 10 microliter analytical flow cell with a 1 cm path length. Data can be acquired up to a rate of 10 per second for a single wavelength and 1 per second for dual wavelength acquisition; 3) Autosamplers – both 48 and 96 sample carousels are available for the 717+ autosamplers. The 2695 Alliance systems have five carousel, turntable autosamplers built-in with the capacity for 120 vials. Sample compartments are temperature controlled 0°C to 40°C. Currently all these systems are validated.
- Gas Chromatography system. This system consists of a Shimadzu GC17A (containing capillary and wide-bore injectors and a flame ionization detector) equipped with an AOC-20 I autosampler and an AOC 20s sample carousel. The system is controlled by Class VP 7 software using a computer similar to those listed above. This system has been validated.
- Bruker Tensor 37 Fourier Transform Infrared spectrophotometer with Near IR and Mid IR spectral ranges, diffuse reflectance sample accessory, and internal sample compartment attenuated total reflectance sample accessory. Instrument operation, data acquisition, and data analysis are controlled by OPUS software. The attached computer is a Pentium 4 1.7 GHz with 256 MB RAM, 20 Gb hard drive with a Windows 2000 operating system. *Capabilities*: Near- and Mid-infrared scanning from any sample accessory, software for spectrum library matching.
- Varian Cary 50 UV/VIS spectrophotometer with temperature control and 18 cell thermostated cell holder (attached to a computer and printer) instrument operation, data acquisition, and data analysis are controlled by Cary WinUV software. *Capabilities*: wavelength range 190-1100 nm; data collection rate 80 points per second. This system is validated of IQ/OQ.
- Beckman Microfuge 22R refrigerated microcentrifuge. *Capabilities*: 24 1.5 to 2.0 mL tube capacity; temperature control from –10 C to 40 C; maximum RCF 18,000 x g with current rotor.
- Anatel Anatoc wide range total organic carbon analyzer (accompanied by an autosampler, attached to a computer and printer). *Capabilities*: The detection range is from 10 parts per thousand to 50 parts per

billion of organic carbon. This system has been validated; autosampler holds up to 63 samples; data are acquired and analyzed by computer.

- The equipment of the Analytical Development Laboratory will be accessible to the Quality Control Laboratory on a need and availability basis. This is a reciprocal agreement requiring both parties to adhere to the highest quality standards required, e.g. cGLP or cGMP.

Microbiology Laboratory, Othmer 321

- Dupont Qualicon Riboprinter automated microbial analyzer is a computer-controlled, DNA electrophoresis-based system that finger prints microorganisms. This system will be used for rapid identification of microbial contaminants. The system can analyze eight samples in eight hours, and additional eight samples sets can be added every three hours.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Formulation Studies – Mark Manning, Ph.D. and William S. Dernell, D.V.M. – Colorado State University Animal Cancer Center

Laboratory:

We have two laboratories within the Animal Cancer Center (ACC), at our disposal. One is an oncology/osteology lab (room 244) and the other a pharmacology lab (room 238). Each lab is approximately 1,200 square feet of space and contains equipment and supplies for tissue culture, drug analysis, formulation work as well as handling and processing blood and tissue samples.

Clinical:

Colorado State University Veterinary Teaching Hospital is one of the premier veterinary clinical sites in the country, with a full range of hospital capabilities. Future directions in our bone research include continuation and expansion of our present work and investigation of additional limb sparing techniques such as isolated limb perfusion and intraoperative radiation.

Animal:

A full-service AAALAC-approved animal facility is available on campus in the Painter Center. This facility has a dedicated surgical suite as well as housing animal care facilities suitable for immunoincompetent animals. Future plans include expansion of the rodent care facilities within the Veterinary Teaching Hospital (close proximity to the ACC) to include immunoincompetent strains.

Computer:

Each member of the Animal Cancer Center has his/her own Pentium-based personal computers with networking capabilities. Laser printers, zip drives, and writable CD drives are available for all key personnel. Computer services within the Clinical Science department maintains and repairs all computer equipment.

Office:

All personnel have either separate or shared office space. The oncology service has a dedicated fax machine as well as a dedicated copy machine. Additional facilities are available through the clinical sciences department.

Other:

The Animal Cancer Center has various meeting rooms for informal lab meetings and seminars, including capability for videoconferencing.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

We have 2 dedicated -80º freezers for long-term storage of tissue and serum samples. These freezers are located in a separate room from our laboratory. Our osteology and pharmacology laboratories have a centrifuge, 2 refrigerators, 2 -20º freezers, a dedicated liquid nitrogen tank for flash freezing and storage, a microscope, an HPLC analyzer, 2 incubators (one CO_2 incubator), 2 water baths, heat block, tissue culture hood, pipets, and glassware. The laboratories are equipped with fume hoods and for chemical storage. The Veterinary Teaching Hospital has histology services available in house through the Veterinary Diagnostic Laboratory.

In addition, Dr. Manning and Dr. Dernell have access to a wide variety of equipment in the Department of Chemistry and the Department of Biochemistry and Molecule Biology for biophysical characterization. This includes mass spectrometry, as well as circular dichroism, infrared, fluorescence spectrometers.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Mouse Assay – Paul E. Monahan, M.D. – University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, NC

Laboratory:

Dr. Monahan has approximately 500 sq ft of laboratory space in Thurston Bowles Hall. This space is a part of the Department of Pediatrics and is located immediately adjacent to the University of North Carolina Gene Therapy Center. This space is equipped with fume hood, thermal cyclers for PCR and for quantitative (real-time) PCR, lyophilizer, Start4 Coagulation Analyzer, Image analysis camera/hardware/software, freezers, clinical centrifuge, water baths, and small equipment for general molecular biology.

Dr. Monahan is an Investigator in the UNC Gene Therapy Center, with total laboratory space approximately 2,300 sq ft, equipped with chemical hoods, super speed centrifuges, cryostat, thermal cycler for PCR, incubators, freezers, and various small equipment for general molecular biology. Also available is a P3 containment facility, 500 sq ft tissue culture facility with four TC hoods, dual stack incubators, a Centra-08R tabletop centrifuge, and microscopes. We also have access to all common equipment in the Gene Therapy Center Viral Vector Core facility, including: controlled temperature rooms (37 degree, 4 degree), dark room, scintillation counter, sonicator, tissue culture room, ultraspeed centrifuge, and glassware facility.

Clinical:

No human subjects work is included in this project. The investigator directs Pediatric Coagulation at UNC-Chapel Hill and is an attending physician at the UNC-CH Comprehensive Hemophilia Treatment Center and the UNC-CH Children's Hospital Pediatric Hematology-Oncology service. He serves or has served as a clinical consultant for Bayer Biologicals and Aventis-Behring in regards to hemophilia clinical care and is/has been an educational consultant for Bayer Biologicals, Baxter Healthcare, and Novo-Nordisk.

Animal:

A virus-free animal facility is located on the first floor of our building. The facility is equipped with a fume and tissue culture hoods, areas for inhaled or injected general anesthesia delivery and care, and procedure/surgery rooms. Basic animal care is provided by qualified animal technicians and a certified veterinarian is available at all times. As an investigator in the NHLBI-sponsored Programs in Excellence in Gene Therapy program, additional care in the selective breeding and screening and generation of novel hemophilia mouse models is assisted through the Transgenic Mouse Core Facility directed by Dr. Randy Thrasher and Dr. Terry Van Dyke.

Computer:

There is access to departmental IBM and Macintosh computers and printers. There is access to the UNC Chapel Hill mainframe, which includes access to Duke University, NC State, and Wake Forest libraries and databases.

Office:

Personal office space is available to the investigator along with administrative, accounting, and secretarial support through both the Gene Therapy Center and the Division of Pediatric Hematology.

Other:

The investigator is a member of the UNC Center for Thrombosis and Hemostasis and the Lineberger Comprehensive Cancer Center. Resources available through the Cancer Center include Oligonucleotide Core Facility, Tissue Culture Core Facility, and DNA Sequencing Core. Resources at UNC-CH available through the investigator's role in the NHLBI-sponsored Programs in Excellence in Gene Therapy program include a Histology/Morphology Core Facility and a Transgenic Mouse Core Facility. Dr. Monahan has long-standing collaborative relationships with Dr. Darrel Stafford, Dr. Timothy Nichols, Dr. Harold Roberts and Dr. Gilbert White and Dr. R. Jude Samulski, who are consulted frequently.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Investigator's laboratory:
Start4 coagulation analyzer
Fibrometer
Microtiter plate reader
Electrophoresis setups
Lyophilizer
Thermal cycler
Quantitative (real-time) PCR

Gene Therapy Center:
Spectrophotometer
Microtiter platereader
Tissue culture hoods and incubators
High and low speed centrifuges

RESOURCES

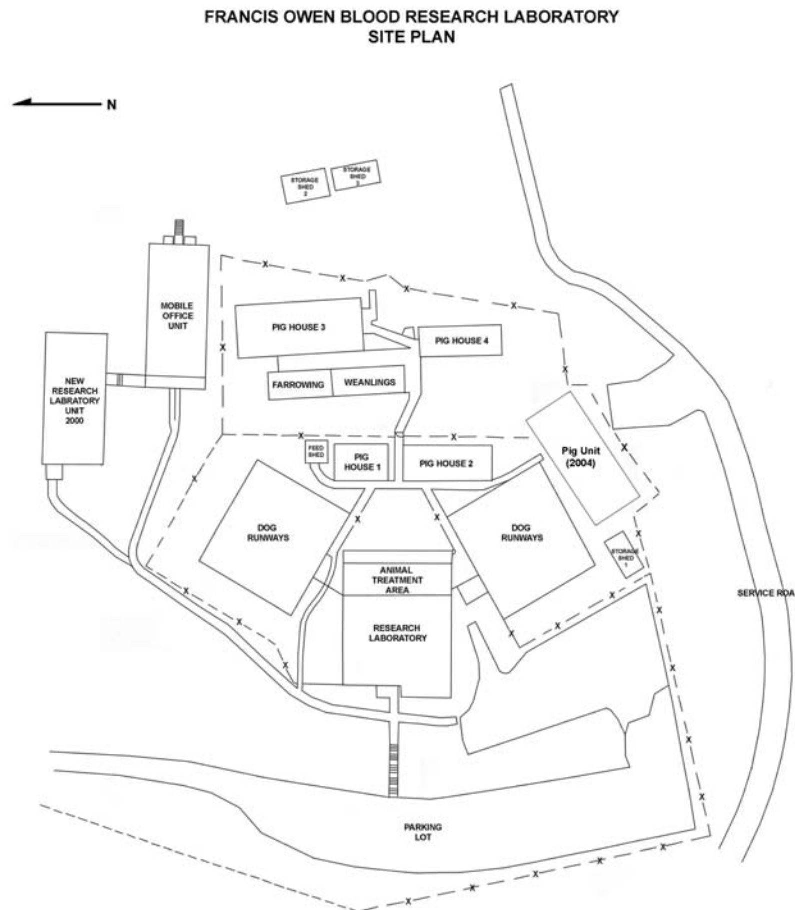
FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Dog Trials – Timothy C. Nichols, M.D. – University of North Carolina at Chapel Hill

Laboratory: The Francis Owen Blood Research Laboratories (FOBRL) was built in 1960 with money from an NIH facilities grant plus matching support from private donors and from the University of North Carolina. The FOBRL is located at University Lake on approximately five acres of University property. The location is four miles from the Medical Center. The property is on a 600+ acre parcel of land that is part of the local water supply. The location is convenient to the medical center, yet relatively isolated from current or potential development of the adjacent private land. The two research laboratories indicated on the attached FOBRL site plan contain 2,400 sq. ft. These laboratories are equipped to perform molecular biology, tissue culture, biochemical studies, protein purification, immunoassays, bioassays, coagulation assays, electrophoresis, and immunohistochemistry. A dark room for film processing and a constant temperature room (i.e., "cold room") are also located within these laboratories. Laboratory bench space is reserved for visiting collaborators to perform bench work on site as needed on a pre-arranged basis.

Clinical: Not applicable.

Animal:



Animal Facilities at the Francis Owen Blood Research Laboratory (FOBRL). The FOBRL has specialized animal housing that meets or exceeds US Department of Agriculture and NIH requirements and AAALAC recommendations. The facility houses strains of dogs with hemophilia B, hemophilia A, von Willebrand disease (VWD), combined hemophilia A/VWD and normal blood donor dogs. The two Dog Runways house the majority of these dogs in indoor/outdoor runs (~5,400 sq ft). Heating and cooling are provided for all animals. In addition, the dogs are allowed into the fenced in area. Special whelping (temperature and humidity controlled) and isolation facilities are available. Pigs with inherited bleeding and metabolic disorders are also housed on site. Normal dogs and pigs with compatible blood types are maintained as a "walking blood bank" from which same-species replacement products are produced. More than 600 transfusions are performed in the animals each year. There is also a support laboratory in the Animal Treatment Area and a diagnostic clinical laboratory located at the FOBRL dedicated to the treatment of these animals.

Computer: Several computer systems are in the laboratories and animal care areas. These computers have comprehensive software for word processing, database management, computer graphics, and internet access via T1 lines to onsite and University servers for all high speed communication and bioinformatics. The University has a dedicated center with equipment and personnel available for microcomputing support.

Office: A 28 x 66-ft modular office building is also located on the grounds of the FOBRL. The building contains office space for the investigators including Dr. Nichols (200 sq. ft), administrative support (200 sq. ft), library, conference room, and a break room for all employees.

Other: The UNC Department of Laboratory Animal Medicine (DLAM) provides all professional veterinary requirements and services. Anatomic pathology laboratories are utilized within DLAM. A suite for performing survival surgery on laboratory animals is located in Berryhill Hall, School of Medicine, Division of Laboratory Animal Medicine. A radiology research laboratory with dedicated X-ray facilities and fluoroscopy is also located in Berryhill Hall. These facilities are available as needed for experiments in the hemophilic dogs.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following equipment is contained within the laboratories at the FOBRL: immunohistochemistry equipment, Perkin Elmer and Beckman spectrophotometers, HPLC, Packard Gamma counter, Molecular Devices Vmax microplate reader, cell counter with emphasis on dog and pig blood analysis (Heska), Thermovac lyophilizer, fraction collectors, absorbance monitors, recorders, pumps, electrophoresis chambers and power supplies, analytical balances, ultra-low freezers, refrigerators, gel dryers, ovens, chemical hood, electrocauterization equipment, Leitz Orthoplan fluorescence microscope, 8 channel physiologic model 7 recorder, Doppler flow crystal and calibration equipment, Sorval JB-4 microtome, micro and high speed centrifuges, incubators for bacteria and tissue culture, 6-foot laminar flow tissue culture hood, PCR machine, water baths, several sizes of electrophoresis systems, PAP-4C Biodata platelet aggregometer, comprehensive gel electrophoresis systems for protein analyses, and ST4 coagulation units

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Stephan B. Abramson, Ph.D. – LifeSci Partners, Altadena, California

Laboratory:
Not Applicable

Clinical:
Not Applicable

Animal:
Not Applicable

Computer:
Pentium 4 PC with 384 MB RAM, 34 GB hard drive storage, CD-RW and DVD-ROM drives, color inkjet, and 600 dpi resolution laser printers.

Office:
Full communication capability, including dedicated phone lines, fax, high-speed internet access.

Other:
Ready access to literature search utilities, several university technical and medical school libraries.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Not Applicable

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under “Other,” identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Julian Cooper, Ph.D. – ProGenetics LLC

Laboratory:

Laboratory at Ripplemead, Virginia: 200 sq. ft. located adjacent to the Swine Research and Housing unit used for preparing the collected milk for storage. Contains wash area, prep area, and milk cooling equipment. Fully accessible for the project.

Laboratory at Blacksburg, Virginia: 1,900 sq. ft. located in Blacksburg, Virginia. Fully functional molecular biology and protein chemistry laboratory, capable of performing standard protocols in DNA manipulation and general protein work. Fully accessible for the project.

Clinical:

Not applicable

Animal:

Swine Research and Housing unit at Ripplemead, Virginia: 900 sq.ft. modular unit with a capacity to hold up to 18 adult animals. Capacity to perform swine milking in a controlled environment. Facility will be fully accessible to the project.

Computer:

One desktop Pentium III computer with ethernet located in the Blacksburg laboratory with full project access, and one desktop Pentium IV computer with DSL located at the Ripplemead laboratory with full project access.

Office:

800 sq. ft. of total office space. 500 sq.ft. available for the project including desk areas, break area, and large conference room.

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Milking crate
Two 30 cu.ft. –40 freezers
Water purification system
Large capacity centrifuge
Microcentrifuge
Spectrophotometer

Research Plan

A. Specific Aims

The hemophilias are congenital disorders characterized by frequent bleeding episodes, especially into the joints and muscles, that cause severe arthritis and crippling. Untreated patients rarely survive past adolescence. The most common hemophilias are caused by congenital deficiency in the clotting proteins Factor VIII (Type A) or Factor IX (Type B), which occur in about 1:5,000 and 1:23,000 male children, respectively. These patients are currently treated with replacement therapy using intravenous administration of purified Factor VIII or IX produced by purification from donor plasma or in cell culture bioreactors. According to a 1995 audit in the state of California, the average healthcare cost for hemophilia patients receiving what is now considered suboptimal care was *$140,000/patient/year-of-life,* of which the overwhelming majority represents the direct cost of Factor VIII or Factor IX replacement therapy. Replacement of clotting factor after a hemorrhage is recognized as suboptimal as some damage still ensues. Optimal therapy consists of regular replacement of the clotting factor to prevent bleeding episodes. Other urgent problems further complicate the clinical situation:

1. Optimal care of patients in the United States is not possible because of the high cost and insufficient supply of clotting factors for replacement therapy.
2. There is currently no access to clotting factors for replacement therapy for 80% of the world's hemophiliacs.
3. Clotting factor shortages occur and are exacerbated by raw material shortages (i.e., plasma) and production plant shutdowns.
4. There is a lack of affordable and adequate amounts of clotting factors for alternative routes of administration, such as oral dosage, which would circumvent the complications and difficulties of intravenous therapy.

Therefore, innovative bioengineering efforts that would provide effective and affordable supplies of replacement clotting factors to treat hemophilia are needed.

The *long-term goal* of this project is to fulfill the objectives of the National Hemophilia Foundation and the World Federation of Hemophilia that call for the provision of worldwide access to prophylactic therapy for both hemophilia A and B. The *objective* of this research is the rapid development of a safe, efficacious, abundant, and inexpensive source of recombinant human Factor IX (transgenic Factor IX) that is ready to be evaluated in clinical trials. Our approach capitalizes on two important features: (1) existing genetically engineered transgenic pigs that synthesize up to 1000 IU/ml (4 grams/liter) of transgenic Factor IX in milk; and (2) favorable pharmacokinetic properties of the transgenic Factor IX in preliminary studies in a hemophilia B mouse model. This unique combination of abundance and quality is the basis of the need for definitive bioengineering research and development that will enable first an intravenous therapy, and subsequently, an oral dosage therapy that can be expected to revolutionize treatment of hemophilia B worldwide.

The proposed research is expected to have a high probability of success because of the scientific soundness and state of development of the basic research, and the strength, breadth, and complimentarity the research team. *ProGenetics LLC* brings a stable, FDA- and USDA-compliant source of transgenic pigs and their milk. Investigators at the *University Nebraska-Lincoln* (UNL) are recognized leaders in bioprocess engineering and current Good Manufacturing Practices (cGMP) production of recombinant proteins for Phase I and Phase II clinical trials (UNL *Biological Process Development Facility - BPDF*), transgenic animal bioreactor engineering, characterization of human Factor IX from transgenic animals, and co-architect of the FDA's *Points to Consider in the Manufacture and Testing of Therapeutic Products for Human Use Derived from Transgenic Animals. Colorado State University* brings expertise in the formulation of stabilized liquid and lyophilized protein samples for oral and injectable delivery. Dr. Stephan Abramson of *LifeSci Partners* has two decades of biopharmaceutical development experience, and has submitted 18 Investigational New Drug (IND)

filings and designed protocols for more than 25 Phase I-IV clinical trials, which led to five new product approvals and four license amendments, including AlphaNine®SD (highly purified plasma-derived Factor IX). The *University of North Carolina at Chapel Hill* (UNC-Chapel Hill) has experience with pre-clinical mouse and dog experimental hemophilia B models and human clinical trials with the only approved recombinant Factor IX produced from cell culture. The *University of Nebraska Medical Center* will serve as the contract research organization to oversee the animal studies at UNC-Chapel Hill in preparation for submission of an Investigational New Drug application with the FDA, which will be the focus of a second grant application to support Phase I clinical studies.

Three *specific aims* are proposed:

Specific Aim # 1. Process engineer and scale-up the recovery and purification of transgenic recombinant human Factor IX. The University of Nebraska-Lincoln Biological Process Development Facility will complete process development and scale-up, and produce clinical grade materials for preclinical studies. The endpoint is a proposed final product specification to help facilitate transfer to *current* Good Manufacturing Practices compliant production of clinical grade material to support an Investigational New Drug filing with the United States Food and Drug Administration (FDA) leading to clinical trials.

Specific Aim #2. Characterize and formulate transgenic recombinant human Factor IX for intravenous dosage, and evaluate in a hemophilia B dog model. These activities are directed toward characterization of the product important to assure the provision of safe and reproducibly effective hemostasis. The results of these investigations will help support an IND filing with the FDA.

Specific Aim # 3. Develop an oral dosage form of transgenic recombinant human Factor IX, and evaluate in hemophilia B mice and dog models. Oral administration of coagulation therapy will obviate the invasiveness, discomfort, potential for opportunistic infection, and complications of storage and supplies that accompany intravenous administration. Oral dosage forms of Factor IX will thus greatly increase the proportion of the patient population that can be treated. There is also published evidence suggesting that oral administration may reduce the potential for complicating immune responses to replacement therapy, especially in patients with severe hemophilia.

Accomplishment of these specific aims will directly address three recommendations in Document #151 of the National Hemophilia Foundation's Medical and Scientific Advisory Committee that are reproduced below:

II. Recommendations to Manufacturers of Coagulation Products

H. Research and development of improved coagulation products that would expedite the transition to total prophylaxis for all persons with coagulation disorders are strongly encouraged.

1. Licensed and improved products to treat patients with von Willebrand disease and patients with inhibitors are urgently needed.
2. Recombinant products that could be taken less frequently or administered other than intravenously would be of tremendous benefit to individuals on prophylaxis regimens.
3. Methods to manufacture coagulation products more inexpensively, such as use of transgenic animals, would increase supply and availability worldwide.
4. Costs of coagulation products should be reduced.

(Revised November 2003, Section II, H, 1-4)

B. Background and Significance

B.1. Significance of the Proposed Research

The high cost of purified hemophilic factor proteins severely limits treatment in the most developed countries to therapy needed when bleeding occurs (Fischer et al., 2002), and, regrettably, little to no treatment for 80% of the world's hemophilia A and B patients in developing countries (Mannucci, 2003). This current therapy is sub-optimal for severe hemophilia but still consumes about 50,000 international units (IU) of Factor IX or more per patient per year (Barrowcliffe et al., 2002) at an average wholesale price of $1.18/IU (Drug Topics Red Book, 2003). The limited supply of donor plasma, the low productivity of cell culture bioreactors, and high development costs of about $800 million for a new therapeutic product (Frantz, 2003) contribute to the high cost of hemophiliac factor proteins. The limited supply also precludes the broad use of prophylactic therapy, which is now regarded as the most effective treatment to reduce the number of bleeding episodes and prevent joint damage (Miners et al., 2000; Van Den Berg et al., 2001; Panicker et al., 2003). As a combined minimum outcome of *Specific Aims 1 and 2*, we expect to engineer an abundant, economical, and potentially long-lasting recombinant Factor IX that is made under *current* Good Manufacturing Practices and is suitable for intravenous delivery and subsequent evaluation in Phase I clinical trials. The successful clinical trial and FDA approval of this product would dramatically increase the availability of prophylactic care in the United States and incidence of treatment elsewhere. We predict that the present work with Factor IX will serve as an important catalyst for progress with making a similar abundant supply of Factor VIII. We are already incorporating knowledge of Factor VIII molecular designs engineered to be secreted at high levels by mammalian cells (Tendulkar et al., 2001) to improve our previous expression in the milk of transgenic pigs (Paleyanda et al., 1997).

An abundant source of a *current* Good Manufacturing Practices-purified Factor IX in the United States would also remove the main obstacle in the development of the most highly desirable drug delivery methods for Factor IX such as subcutaneous, intramuscular, intratracheal, and oral administration. Currently, all Factor IX products are given intravenously to attain maximal bioavailability. Importantly, oral administration of Factor IX in dogs (Horikoshi et al., 1982) and Factor VIII in both dogs and humans (Hemker et al., 1980; Horikoshi et al., 1982) is possible, but the low bioavailability makes this mode of treatment impossibly expensive with respect to the limited supply of current Factor IX sources. We are confident that the abundance of a highly purified recombinant human Factor IX and knowledge from the pharmacokinetics of its intravenous administration derived from a successful completion of *Specific Aims 1 and 2* will enable us to engineer an optimal Factor IX oral dosage for evaluation in preclinical animal models. Successful completion of *Specific Aim 3,* especially in the hemophilia B dog model, will help to justify future human clinical trials. Thus, the ultimate goal would be to make an abundant oral dosage form of Factor IX that simplifies treatment of patients worldwide, especially of hemophiliac infants. Because oral administration has already been reported using plasma-derived Factor VIII (Hemker et al., 1980), a similar advance in hemophilia A treatment can also be catalyzed by the success of the present work with Factor IX.

B.2. Review Of Literature That Is Relevant To This Application

B.2.1. Fundamental biochemical constraints for producing recombinant human Factor IX. We here explain why Factor IX is a scarce protein therapeutic by first describing the relationship between its structure-function complexity and the difficulty in making it. Secondly, we discuss the structure-function standards needed to define the goals of the proposed work. **Figure 1** shows the complex molecular structure of Factor IX. Factor IX plays a critical role in the blood-clotting enzymatic cascade that results in the formation of a blood clot to restore hemostasis (Kurachi et al., 1993). Briefly, it circulates as a complex precursor protein (i.e., zymogen) at a concentration of 5 μg/ml in plasma. In response to a bleeding event, Factor IX is activated by either Factor XIa or Factor VIIa/Tissue Factor to release a 35 amino acid activation peptide (Ala146 to Arg180). Activated Factor IX (FIXaβ) then acts in

concert with Factor VIIIa to proteolytically activate Factor X, which, in turn, activates prothrombin to thrombin. Thrombin cleaves fibrinogen to fibrin, and fibrin polymer forms the clot.

The biological activity of Factor IX requires a complex molecular structure that contains post-translational modifications. Factor IX is a member of the Vitamin K-dependent family of proteins, which are distinguished by the post-translational γ-carboxylation of glutamate (Glu) to γ-carboxyglutamate (Gla). This post-translational modification requires Vitamin K as a cofactor and the Factor IX propeptide (Figure 1) as the binding site for the carboxylase enzyme complex (Suttie, 1993; Furie et al., 1997; Wu et al., 1997). After the N-terminal glutamates are γ-carboxylated, the propeptide is removed. Both of these two molecular processing steps are required for biological activity (reviewed in Freedman et al., 1996; Sunnerhagen et al., 1996). In terms of a therapeutic product specification, Factor IX products must have at least 6 γ-carboxyglutamates/molecule to be functional (Kaufman et al., 1986; Lindsay et al., 2004 [see Appendix]), and the existing recombinant Factor IX product, BeneFIX®, has 10-12 (Gillis et al., 1997, White et al., 1998). The complexity of these post-translational modifications necessitates production of biologically active Factor IX in mammalian cells. Furthermore, only certain mammalian cells are capable of making these post-translational modifications at protein synthesis rates sufficient for commercial scale production. Very few mammalian cells can efficiently γ-carboxylate and also subsequently remove the propeptide.

BeneFIX® is the only FDA-approved recombinant human Factor IX (transgenic Factor IX), and it is produced in Chinese hamster ovary (CHO) cells. We review the molecular, functional, and production engineering characteristics of BeneFIX® because it serves as the key reference for the transgenic recombinant human Factor IX proposed here. Briefly, BeneFIX® contains a mixture of sub-populations with 10, 11, and 12 γ-carboxyglutamates (White et al., 1998). This is consistent with the reported limitations in the ability of Chinese hamster ovary cells to γ-carboxylate Factor IX (Kaufman et al., 1986). The problem of having a mixture of biologically active and inactive Factor IX populations was solved by designing a purification process consisting of four chromatographic steps designed to purify and select for γ-carboxylated transgenic Factor IX (Harrison et al., 1998). Another salient feature of BeneFIX® is that, unlike plasma-derived Factor IX products, it contains essentially no phosphorylation at Ser-158, and only 15% sulfation of Tyr-155 (Bond et al., 1998). Pharmacokinetic studies in humans indicate that the lack of phosphorylation and sulfation may lead to a 33% lower initial recovery of activity in plasma after intravenous infusion for BeneFIX®, as compared to plasma-derived Factor IX (White et al., 1998; Berntorp and Bjorkman, 2003). Other post-translational modifications made to Factor IX include β-hydroxylation Asp-63, two N-linked glycosylation sites at Asn-157 and Asn-167 within the activation peptide, and four O-linked glycosylation sites at Ser-53, Ser-61, Thr-159, and Thr-169 (Figure 1). The specific contributions of these post-translational modifications to Factor IX biological activity have not yet been determined. However, N-linked oligosaccharides of the "complex" designation that contain terminal sialic acid monosaccharides have been shown to significantly improve the circulation half-life of therapeutic proteins (Varki et al., 1999). Plasma-derived Factor IX has complex N-linked oligosaccharides that are tri- and tetra-antennary and terminated with sialic acid (N-acetylneuraminic acid, Makino et al., 2000). N-linked oligosaccharides of BeneFIX® were similar to plasma-derived Factor IX (Bond et al., 1998). In summary, our basis

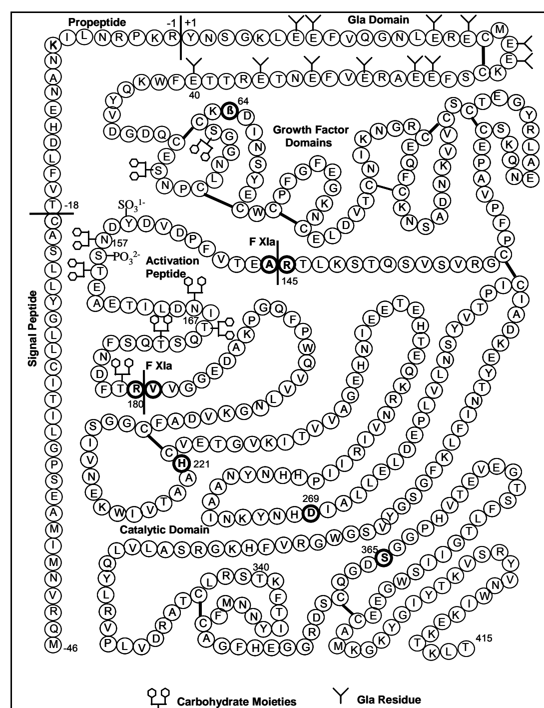


Figure 1. Factor IX amino acid sequence, indicating the propeptide (Thr[-18]-Arg[-1]), post-translational modifications in the "Gla domain" (Tyr1-Glu40), and the activation peptide (Ala146-Arg180).

for determining product specifications suitable for the transgenic recombinant Factor IX will utilize the above-mentioned molecular, biological activity and process engineering knowledge base provided by BeneFIX®.

B.2.2. Transgenic mammary tissue and the advantages of pig mammary tissue to produce human recombinant Factor IX. We here discuss the reasons why the milk of the transgenic pig can be a prodigious and expedient source of recombinant Factor IX relative to cell culture bioreactors. Transgenic animals already have been used to produce many recombinant proteins that are secreted into milk at a research and pilot plant level (Rosen et al., 1996; Lubon, 1998; Clark, 1998; Houdebine et al., 2000). The reason why mammary cells make high concentrations of milk proteins is due to their high cell density, about 10^9 cells/ml, which results in protein production rates of about 1-15 grams/liter/hour. This cell density is two to three orders of magnitude greater than what is possible in mammalian cell culture production such as used to make BeneFIX® and other complex recombinant therapeutic proteins. The specific choice of using the pig relative to other milking livestock is based upon the combination of high volume of milk that can be harvested per day (2-3 liters) and the quality of post-translational processing of complex proteins. **Figure 2** shows a transgenic pig being milked by a pig milking machine that typically yields about 0.4 to 1.25 liters per milking. Pigs can be milked up to five times per day over two 50-day lactations per year, and so the total milk yield from the average sow is 200-300 liters/year.

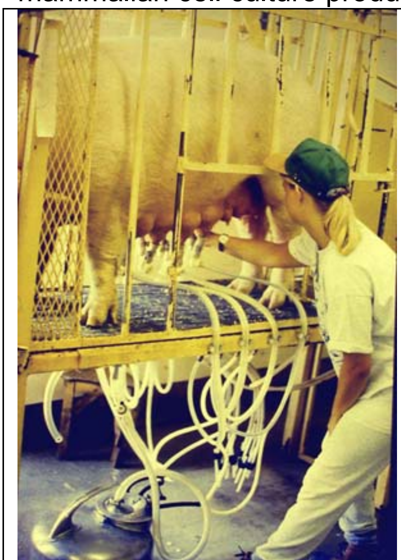


Figure 2. Lactating sows have a milk letdown every hour during daylight hours and can be milked 3-5 times/day. About 2-3 liters/day/sow can be collected over two 50-day lactations/year. Milk can be collected by hand or by machine as shown.

The current scarcity of Factor IX protein is predictable due to the low concentrations in both human plasma (5 μg/ml) and animal cell culture bioreactors (~ 10 μg/ml). Presently, less than 10% of the United States hemophilia B patients receive prophylaxis. The projected process volumes needed to scale-up to satisfy the clinical demand in the United States translate to large capital investment costs because the facilities to produce and process these source materials do not currently exist (Garber, 2001). **Figure 3** on the next page compares the amounts of Factor IX source materials needed to meet the demand for prophylactic therapy for the estimated 3,000 patients at a level of 200,000 IU/patient/year. More than 1 million liters of plasma per year would be required to satisfy this demand. An animal cell culture bioreactor facility producing approximately 600,000 liters of supernatant/year and costing in excess of about $50 million capital investment would be required to generate a similar amount of Factor IX. In contrast, using milk from existing transgenic pigs (conservatively, 100 IU/ml), *only 60 pigs would be needed supply prophylactic treatment to the 3,000 patients in the United States.* The capital investment for this pig production facility is less than $2 million, and importantly, the marginal cost for scaling up transgenic animal production is very low compared to animal cell culture bioreactor facilities. This is the key advantage of using transgenic pigs for rapidly achieving an abundance of economical Factor IX – the costs for purification per unit of material are similar between mammalian cell culture and transgenic bioreactors.

In addition to the consideration of milk volume, the mammary epithelial cells of the pig are unique among livestock in making complex post-translational modifications needed for Factor IX biological activity. From a broad biochemical and physiological perspective, the pig is more closely related to humans than the ruminants, which include the cow, sheep, and goat. As an example of the differences between ruminant and pig biochemistry, recombinant Factor IX production in sheep was unsuccessful with only low levels of biologically active Factor IX being produced – less than 1 nanogram of biologically active Factor IX could be recovered from each milliliter of milk (Clark et al., 1989); the sheep was even less productive than donor plasma. In contrast, porcine mammary epithelial

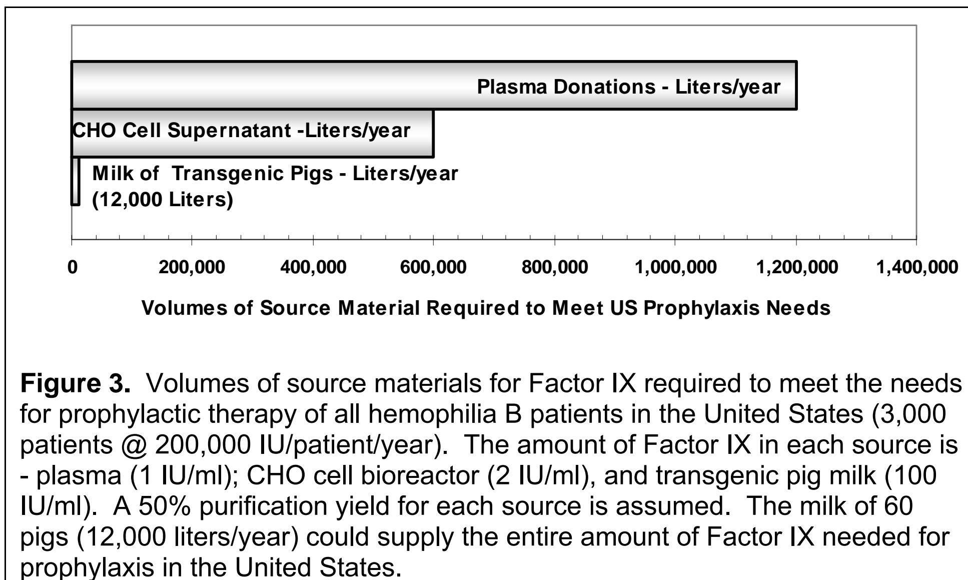


Figure 3. Volumes of source materials for Factor IX required to meet the needs for prophylactic therapy of all hemophilia B patients in the United States (3,000 patients @ 200,000 IU/patient/year). The amount of Factor IX in each source is - plasma (1 IU/ml); CHO cell bioreactor (2 IU/ml), and transgenic pig milk (100 IU/ml). A 50% purification yield for each source is assumed. The milk of 60 pigs (12,000 liters/year) could supply the entire amount of Factor IX needed for prophylaxis in the United States.

cells have been found to be capable of making the post-translational processing required for making biologically active Protein C (Velander et al., 1992), a Vitamin K-dependent protein homologous to Factor IX, and we have recently confirmed this result in recombinant human Factor IX (Van Cott et al., 1999 and Lindsay et al., 2004). In addition, the N-linked glycans added to the endogenous porcine milk glycoprotein lactoferrin are complex glycans terminated with N-acetylneuraminic acid (sialic acid), which is needed for long circulation half-life of therapeutic proteins. In contrast, ruminant lactoferrins contain undesirable high-mannose glycans (Spik et al., 1994). Thus, ruminants will likely not be able to make a recombinant Factor IX having glycosylation needed for long half-life. In summary, we propose that the combination of the abundance and quality of the recombinant Factor IX made by the transgenic pig mammary gland will be the most important features needed for future development.

B.2.3. The safety requirements for FDA approval of a transgenic pig supply of recombinant human Factor IX. We here review the knowledge base needed to anticipate regulatory concerns and make production specifications to assure the safety of products derived from transgenic pigs. In recognition of the feasibility of this production method, the United States FDA has issued a document addressing the *Points to Consider in the Manufacture and Testing of Therapeutic Products for Human Use Derived from Transgenic Animals*. Importantly, pioneering work on the safety and efficacy of recombinant anti-thrombin III produced in the milk of transgenic animals has been demonstrated in clinical trials by Genzyme Transgenics Corporation (Levy et al., 2001). To reduce risks of disease transmission from milk, multiple barriers are placed in the purification process that provide logs of pathogen removal and/or inactivation of common classes of lipid envelope and non-lipid envelope viruses: chromatography (Hay, 2002), solvent/detergent treatment (Horowitz et al., 1992), and nanofiltration (Van Holten et al., 2002). Maintenance of specific pathogen-free production herds is also an important requirement. Many of these steps are currently in place for many human plasma-derived Factor IX products, animal-cell-derived products such as BeneFIX®, and animal products such as heparin and porcine Factor VIII (Hyate:C). The process technology for implementing these steps with transgenic-animal-derived products has been demonstrated to be directly transferable (Ziomek, 1996).

Clinical experience with the porcine-derived Factor VIII product called Hyate:C is instructive for assessing and reducing the risks involved in zoonosis from porcine-derived therapeutic protein products. Hyate:C is a product alternative used to treat hemophilia A patients that develop an immune response to plasma-derived or recombinant-derived human Factor VIII. These patients produce antibodies that neutralize the activity of these Factor VIII products. Hyate:C was originally produced using largely unregulated slaughterhouse pigs and has been given to patients since 1984 without any viral inactivation steps in the purification process. In 1996, detection of porcine parvovirus (PPV) DNA in multiple batches halted production (Soucie et al., 2000). In a comprehensive retrospective study of hemophiliacs receiving Hyate:C prior to 1996, it was concluded there was not a single case of transmission of a porcine virus to a patient receiving Hyate:C (Giangrande et al., 2002). Thus, the threat of one of most common pathogens that occurs in pigs has been evaluated. Another example of

a safety concern for parenteral therapeutics is transmissible spongiform encephalopathies such as BSE. It has been recently shown that swine are resistant to nutritional challenges of highly infective tissue homogenates from BSE-infected cattle (Wells et al., 2003). In addition, specific pathogen-free transgenic production herds are fed only plant-derived diets.

Additional safety issues are specific to the biochemistry and physiologic action of Factor IX. These safety issues have been well addressed in both plasma-derived and recombinant Factor IX products that are currently available. The primary issues concern the presence of activated Factor IX species that could induce hypercoagulopathy, a dangerous phenomenon caused by excessive blood clotting usually occurring immediately or shortly after infusion. Most product specifications meet this safety requirement by using purification processing and formulations that minimize the presence and generation of activated Factor IX during storage. The presence of heparin contamination could also lead to bleeding events caused by suddenly depressed platelet cell count. While many plasma-derived Factor IX products contain heparin, increased safety can be likely be attained by minimizing heparin content arising from heparin affinity chromatography. The above mentioned process technology should be directly applicable to a recombinant human Factor IX product derived from transgenic pig milk.

B.2.4. Alternative delivery routes for Factor VIII and Factor IX enabled by abundant supply.

B.2.4.1. General extravascular Factor IX delivery alternatives. We here discuss alternative delivery methods that might be made possible due to the abundance of purified transgenic Factor IX supply made possible as a result of successfully completing our research objectives. Hemophilia clinicians treating patients in economically depressed locations have stated that ensuring patient compliance would likely be improved if alternatives to intravenous infusion could be implemented (Dr. Carol Kasper, personal communication). These delivery alternatives differ from intravenous infusion with respect to the balance between ease of administration and bioavailability. It has previously been demonstrated that Factor IX can be transported across endothelial and epithelial cell layers. For example, aerosolization of Factor IX may provide an alternative, needle-free therapeutic option for delivery of Factor IX across the epithelial cell layer in the lungs (Russell et al., 2001). This recent study demonstrated that bioavailabilities of 11% (relative to intravenous administration) were obtained in hemophilia B dogs using 200 IU/kg-bodyweight intratracheal dosing regimen. The pharmacokinetic results were consistent with a slow release from a deposition site in the lungs. The intra-tracheal administration produced therapeutic levels of both antigen and activity, and this activity was detected through 72 hours post-administration. In addition, there has been a group of studies to determine the absorption rate and bioavailability of plasma-derived Factor IX administered through other extravascular routes: subcutaneous, intramuscular, and intraperitoneal (Liles et al., 1997; McCarthy et al., 2002). While all of these routes resulted in measurable Factor IX activity, the subcutaneous and intramuscular injections resulted in bioavailabilities of 63.5% and 82.8%, respectively (Liles et al., 1997). Subcutaneous administration of high concentrations of up to 4000 IU/ml resulted in bioavailabilities of 35% in cynomolgus monkeys and 46% in hemophilia B dogs (McCarthy et al., 2002). Importantly, while all of these extravascular delivery routes are less efficient than intravenous, they provide a combination of simplicity of administration and sustained Factor IX activity. Thus, if an abundant and economical source of Factor IX were available, then the advantage of clinical simplicity becomes more of an important and feasible consideration.

B.2.4.2. Oral dosage formulations. Oral dosage form of Factor IX may provide the most facile and convenient delivery of Factor IX. In general, low bioavailabilty of oral protein dosage forms arise from degradation within the gastrointestinal environment and the resistance of high molecular weight proteins to be transported through the mucus gel layer and epithelia and into the bloodstream (Heizmann et al., 1996; Bernkop-Schnurch et al., 1999). The inefficiency due to the gastrointestinal environment can be mitigated by the following: modifying the pH of the stomach; competitively inhibiting pancreatic proteases with inhibitors such as soybean trypsin inhibitor, aprotinin, or casein; and high dosage regimens (Anderle et al., 2002). However, even with optimal formulations, high oral dosage

may provide the lowest bioavailability of the alternative delivery formulations. Thus, oral dosage is feasible only when abundance and cost are favorable and low patient plasma levels are needed to achieve effective therapy. A low bioavailability may also be more feasible in children and infants, where total body weight is low and intravenous delivery is more problematic. In the case of Factor IX and Factor VIII, only about 5% of normal plasma levels are needed to maintain normal hemostasis. This translates to only about 250 and 12 nanograms per milliliter of plasma for Factor IX and Factor VIII, respectively. These levels have been achieved in human and dog plasma levels using liposome and enzyme inhibitor formulations of oral dosages of human plasma-derived Factor VIII and Factor IX (Hemker et al., 1980; Horikoshi et al, 1982). Since both Factor VIII and Factor IX naturally adsorb to cell surfaces, these data suggest that once delivered to the intestinal mucosal surface, these proteins would be amenable to an absorption process that could result in passage into the bloodstream.

Hemker et al. (1980) used Factor VIII formulated in liposomes, administered a dose of 290 Units to a hemophilia A adult patient, and obtained greater than 5% Factor VIII activity in plasma for 50 hours after dosing (**Figure 4**). The profile is consistent with a slow absorption process resulting in a broad plasma level response. In the case of hemophilia, where clotting factor levels of only 0.05 IU/ml (which is 5% of the activity in normal plasma) are needed for normal hemostasis, a broad plateau of 5% activity or greater sustained over more than 48 hours is desirable. Horikoshi et al. (1982) also used a liposomal formulation and administered a dose of 14 Units of plasma-derived Factor IX/kg bodyweight in a 9.2 kg dog and measured 125% of normal Factor IX activity by aPTT. In both these Factor VIII and Factor IX oral dosage studies, however, sufficient numbers of animal or human subjects were not investigated to assess the variability in bioavailability that would be expected using oral dosage. We assume that at that time, the supply of plasma-derived materials precluded the effective parametric study of appropriate formulations to determine optimum bioavailability. In the following section, we will also review our success in oral dosage of transgenic recombinant Factor IX to adult mice and dogs.

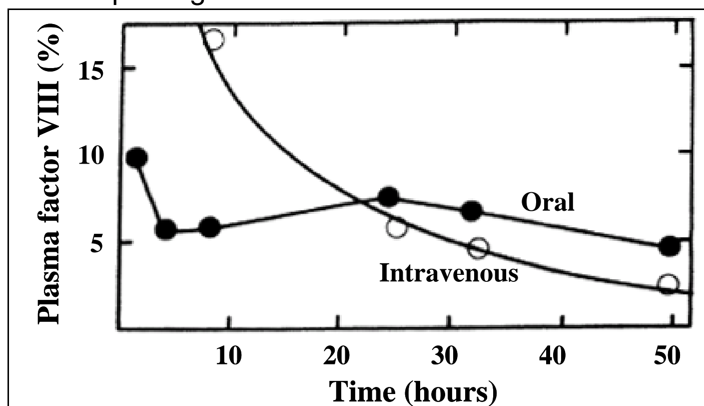


Figure 4. Reproduction of the results from Hemker et al. (1980): "*Factor VIII activity in plasma after oral and intravenous administration of Factor VIII to a patient with severe hemophilia. Concentrations... are expressed as percentage of Factor VIII concentration in pooled normal plasma (n=30). Each sample was tested four times.*"

In summary, there is mounting evidence in humans and in experimental animals that suggests intact therapeutic proteins can be absorbed from the gut into plasma (Mayer, 2003). In addition to the above examples, the absorption of biologically active protein from the gut to plasma has been demonstrated with oral administration of erythropoietin in 10-day-old rats (Miller-Gilbert et al., 2001) and TGFβ1 in neonatal mice (Letterio et al. 1994). In the context of these experiments, we propose to supply an abundant source of recombinant human Factor IX, which, under more optimal formulation, should minimally prove useful in the treatment of infants where only small amounts of units will be needed to achieve prophylaxis due to small body weight.

B.3. Important Conclusions From Knowledge Base About Current Factor IX Supplies and Production Methods

The relationship of the structure and function of Factor IX requires it be made in a mammalian cell. The pig mammary epithelial cell can effectively make the post-translational modifications needed for a functional, recombinant human Factor IX. The high cell density of the mammary gland enables prodigious concentrations of Factor IX to be made in the milk. The estimated milk volumes will enable a rapid scale-up to production levels of Factor IX that would meet clinical demand for prophylactic

treatment that is not now being met by conventional animal cell bioreactor technology. The abundance of Factor IX produced by the pig would make feasible alternative delivery methods that are greatly more desirable than intravenous delivery, especially for infants and small children.

C. Preliminary Studies

C.1. Description of the Transgenic Pigs: Molecular Genetics, Specific Pathogen-Free Pedigree

C.1.1. Molecular genetics of transgenic Factor IX pigs: genotype and phenotype. We here present preliminary data that describe the existence of transgenic pigs that produce high levels of Factor IX in their milk. We have used two regulatory motifs utilizing genetic elements from the murine Whey Acidic Protein gene to express two levels of transgenic Factor IX cDNA. We previously reported the high-level expression of transgenic Factor IX at 0.1-0.2 grams/liter in the milk of transgenic pigs using a 2.5 kbp murine Whey Acidic Protein promoter (Van Cott et el., 1999). We have recently made transgenic pigs containing a murine Whey Acidic Protein regulatory element consisting of 4.1 kbp of 5' promoter region. This construct directs mammary-specific expression of transgenic Factor IX at 3-4 grams/liter in milk. This transgene construct uses no viral DNA elements.

Two different genotypic signatures have been detected through fourth generation outbreeding of a single founder transgenic male made by microinjection. In preparation for microinjection, the construct was excised from the plasmid by Not I restriction digest and extracted and purified to remove vector contaminants. The propagation of the original transgenic founder male is shown in **Figure 5**. Pigs have been characterized by PCR and Southern Analysis for head-to-head, tail-to-tail, and head-to-tail transgene content. The genotype designated A contains a head-to-head integration within a single integration site of about eight transgene copies. This head-to-head site is spontaneously deleted to form a B genotype. The B genotype has not shown this instability.

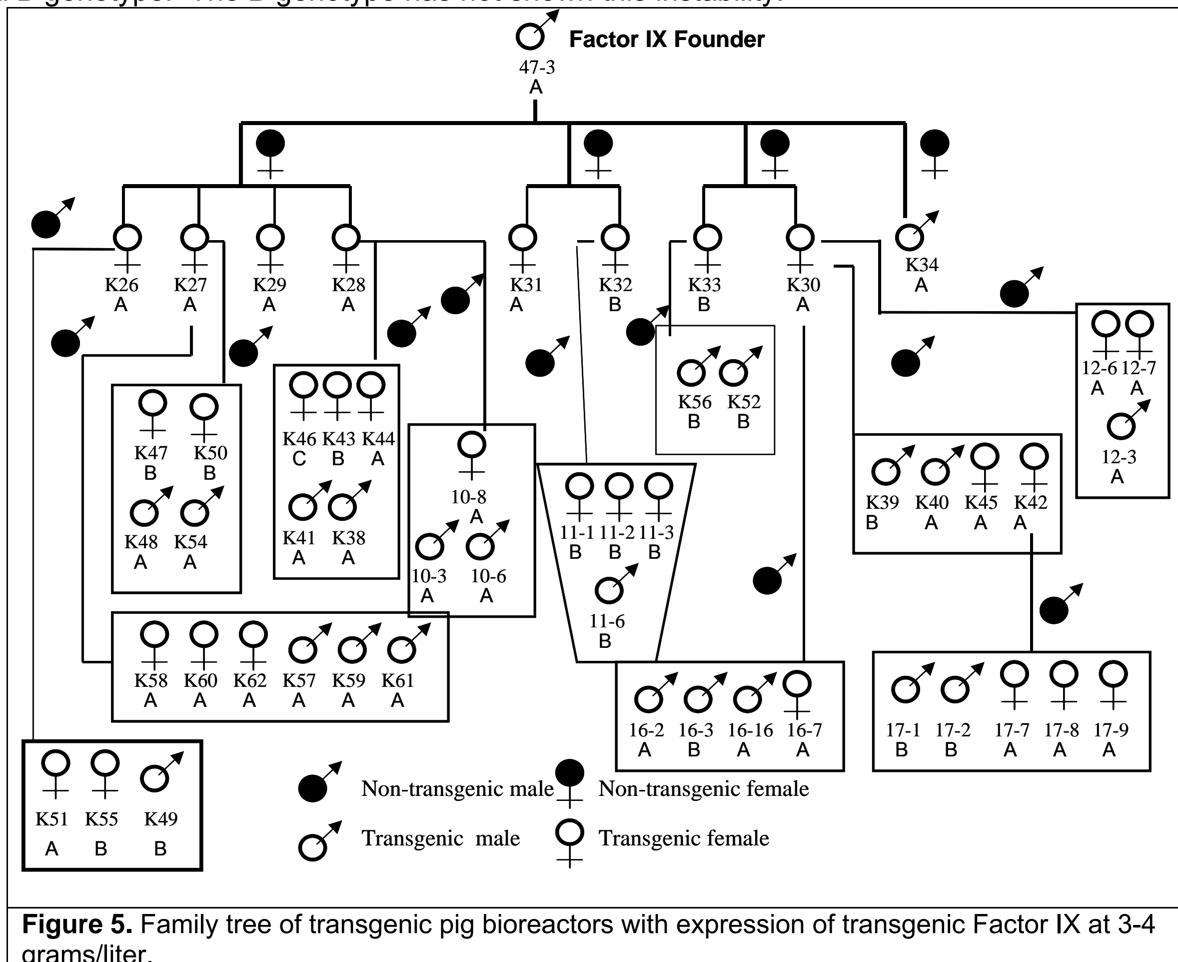


Figure 5. Family tree of transgenic pig bioreactors with expression of transgenic Factor IX at 3-4 grams/liter.

The phenotype of these animals lends itself to an engineering analysis of transgenic Factor IX throughput. In particular, the phenotype of both A and B genotypic animals appears to be the same with both genotypes exhibiting a constant expression of the recombinant human Factor IX at 3-4 grams/liter throughout a lactation of up to 70 days. The amount of Factor IX activity in the milk ranges from 100-1000 IU/ml; because Factor IX has a theoretical activity of 200 IU/mg, some Factor IX being produced by the pig at these high levels is not biologically active. We have found that Vitamin K nutritional supply and Vitamin K bioavailability to the mammary gland significantly impact the extent of γ-carboxylation of transgenic Factor IX, and, hence, the amount of biologically active transgenic Factor IX that is produced. ProGenetics will supply milk from animals who have had already have an optimized regimen of Vitamin K nutritional supplementation. The team at the University of Nebraska-Lincoln will determine the variability of γ-carboxylated product and respective specific activities within defined product milk pools. Thus, a key task will be to define the milk pool specifications that will give a reproducible product quality in terms of specific activity and reproducibility.

C.1.2. Specific pathogen-free pedigree of currently available transgenic Factor IX pigs. The suitability of the transgenic pigs to be used in this proposed research for materials intended to eventually be used in clinical trials derives from their specific pathogen-free pedigree and the maintenance of biosecurity at ProGenetics' facilities. ProGenetics maintains fourth generation transgenic animals (ProGenetics FDA INAD filing no. 11-045) and a semen bank at a restricted access, commercial research animal facility located in the mountains of southwest Virginia. The mountains of southwest Virginia are a low livestock animal density location virtually free of standing water, and so there is also a low density of biting insect vectors. This facility is in compliance with currently accepted FDA and USDA practices. Under the guidelines in *NIH Guidelines for Research Involving Recombinant DNA Molecules* (April 2002) the transgenic swine fall under the category of biosafety level 1 (BL1-N animals). Current facilities at ProGenetics fulfill all requirements established in Appendix Q sub headings Q-I-B-2 through Q-II-A-2-a for such BL1-N animals. Any future facility for housing the animals would meet or exceed the same guidelines. Transgenic animals are permanently identified by tattoo and ear notch along with semi-permanent identification by ear tagging. An animal record is maintained where each animal identification record contains date of birth, sex, genotype, and complete health history. Each animal record is contained in a database consisting of both electronic and paper forms. Also included in the record, when timely, will be the date of death and cause, if known.

The specific pathogen-free pedigree of the transgenic pigs has been determined by ProGenetics, and the herd is free of Brucellosis, Pseudorabies, and porcine Parvovirus. The Commonwealth of Virginia is Brucellosis-, Tuberculosis- and Pseudorabies-free. Nine sentinel animals have been recently screened for Porcine Reproductive and Respiratory Syndrome (PRRS) and Transmissible Gastroenteritis and found to be negative. There has not been a case of swine dysentery (vibrionic dysentery), salmonellosis, erysipelas, transmissible gastroenteritis, PRRS, porcine encephalomyocarditis, aino and akabane viruses, pseudorabies, or enzootic polyserositis in either this herds or in any herds within 10 miles of the facilities. These animals were born and raised in the United States and have not been fed garbage or rendered animal products at anytime. The United States is currently validated as free of Foot and Mouth Disease, Akabane Virus, African Swine Fever, Classical Swine Fever, Swine Vesicular Disease, Japanese Encephalitis and Teschen's Disease, which are considered serious diseases of zoonotic origin from pigs to humans.

Transgenic females are artificially inseminated with semen from a commercial semen supplier (Birchwood Farms, OH) that is certified to be free of Pseudorabies, PRRS, Brucellosis, SIV, and mycoplasma. Transgenic boars are bred to nontransgenic females purchased from commercial facilities that are certified free of Pseudorabies, porcine parvovirus, PRRS, Brucellosis, SIV and mycoplasma, such as those provide by P.I.C.(Franklin, KY).

C.2. cGMP Production of Biologicals at the University of Nebraska-Lincoln Biological Process Development Facility

The University of Nebraska-Lincoln Biological Process Development Facility is a turn-key academic process development facility with a full-time staff of 36 scientists and technicians dedicated to research and development and the rapid transition of human biologics into Phase I clinical testing. It is capable of producing Phase I injectables and Phase II oral biologics at the 500 L bioreactor-scale (http://bpdf.unl.edu). It requires 9 to 12 months to develop a scalable process for *current* Good Manufacturing Practices (cGMP) manufacturing and 2 to 3 months to produce Phase I clinical material. The Biological Process Development Facility recommends technology transfer to a commercial partner between Phase I and II, and continues to produce early Phase II material until technology transfer is completed.

The Biological Process Development Facility has eight groups: molecular biology, fermentation development, cell culture development, purification development, analytical methods development, quality control (analytical and microbiology), cGMP manufacturing (fermentation and purification pilot plant), and quality assurance. It has worked with 25 companies over the last 10 years on 29 clinical products. The Biological Process Development Facility has been under contract with the United States Army Medical Research and Materiel Command on the botulinum and ricin vaccine and human antibodies against botulinum. Recently, Dynport Vaccine Company was awarded $11 million to fast-track the development of a heptavalent vaccine against botulinum of which $6.5 million was awarded to the University of Nebraska-Lincoln. In addition, the Biological Process Development Facility is producing at the 500 L scale recombinant protein for Phase II oral studies and was responsible for all aspects of process development. It has successfully completed Quality Assurance audits by Cato Research (8/02/03) and Bioprocess Consultants (9/15/03) for early phase cGMP production agreements. The Biological Process Development Facility has the experience to develop, transition, and produce cGMP transgenic recombinant Factor IX for clinical trials.

C.3. Molecular Characterization of Factor IX Produced in the Milk of Transgenic Pigs

Factor IX, whether purified from human plasma or produced in Chinese Hamster Ovary cells, exists as a spectrum of subpopulations that differ in the nature and extent of post-translational modifications. The fundamental challenge to the bioengineer is to develop a product specification that results in a safe and efficacious product and a process that can reproducibly make the specified product.

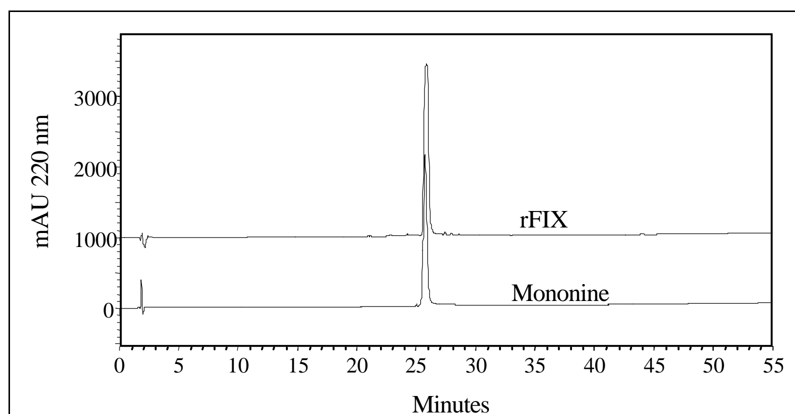


Figure 6. Recombinant Factor IX from the milk of transgenic pigs can be purified to high purity by heparin-affinity chromatography, as shown by reverse-phase HPLC analysis of the product (Lindsay et al., 2004). For comparison, plasma-derived Factor IX purified by immunoaffinity chromatography (Mononine®) was also analyzed.

We have purified Factor IX from two lineages of transgenic pigs at the laboratory bench scale (Van Cott et al., 1999; Lindsay et al., 2004). The design of the purification process for the lineage producing 3-4 grams/liter was to capture the majority of sub-populations that occur in the milk so that they could be studied. Skim milk was first treated with ethylenediaminetetraacetic acidic (EDTA) to dissolve casein micelles and micelle-adsorbed Factor IX prior to application to a heparin-affinity chromatography column. Starting with milk having 150 U/ml of Factor IX activity, a product activity yield of >95% was obtained at a purity of >98%, as judged by HPLC and sodium dodecyl sulfate

polyacrylamide gel electrophoresis (**Figure 6**). Once this pure product was obtained, the subpopulations of the recombinant Factor IX were fractionated by high-resolution anion exchange chromatography. In Lindsay et al. (2004; see Appendix), we used gradient elution from anion exchange columns to selectively purify the recombinant Factor IX populations. We produced a Factor IX product having a biological activity that was 200 IU/mg, similar to that of plasma-derived Factor IX. We assumed a reference Factor IX concentration in plasma of 5 μg/ml and an activity level of 1 IU/ml and, therefore, a reference specific activity of 200 IU/mg of Factor IX.

We have begun preliminary structural analysis of the biologically active transgenic Factor IX. While sialic acid content in N-linked and O-linked glycans is not linked to enzymatic activity (Bharadwaj et al., 1995), in general, it can have a significant impact on pharmacokinetics as de-sialylated proteins are rapidly cleared by receptors in hepatic cells (Goochee and Monica, 1990). Plasma-derived Factor IX has complex N-linked glycans that are tri- and tetra-antennary and terminated with N-acetylneuraminic acid (NANA) (Makino et al., 2000). The sialic acid content of transgenic Factor IX was measured by the Warren assay (Warren, 1959) and by fluorescent labeling and capillary electrophoretic separation/detection (Chen et al., 1998). Using both methods, 6-9 moles NANA/mole transgenic Factor IX were measured. Importantly, the sialic acid was confirmed to be NANA by capillary electrophoresis, and not N-glycolylneuraminic acid (NGNA), which is commonly added to recombinant proteins produced in transgenic goats (Edmunds et al., 1998).

The phosphorylation of Ser-158 in Factor IX is thought to be an important determinant of pharmacokinetic properties (White et al., 1998). BeneFIX® is produced in Chinese Hamster Ovary cells and has essentially no phosphorylation (Bond et al., 1998) while plasma-derived Factor IX products such as Mononine® and AlphaNine®SD have one phosphoserine/molecule. Using a colorimetric phosphorylation assay from Pierce Chemical (Rockford, IL), we measured two phosphoserines/molecule in transgenic Factor IX, and one phosphoserine/molecule for Mononine®. Mammary epithelial cells have an enormous capacity for phosphorylating peptide sequences having a motif of Ser-X-Asp/Glu motifs in the endogenous caseins that are secreted at a level of 30-40 grams/liter (Alexander et al., 1992), and Factor IX has four of these sequence motifs, including one in the γ-carboxyglutamate domain (Figure 1).

C.4 The Hemophilia B Mouse Model Used to Evaluate Hemostatic Effects of Factor IX and its Pharmacokinetic Properties

We here discuss preliminary data that help to show the potential for using transgenic recombinant Factor IX as a post-bleeding event treatment and as a therapeutic for prophylaxis. We used hemophilia B mice that have a gene sequence knock-out that prevents Factor IX antigen from being produced, and, therefore, represents an animal model that is Factor IX cross-reactive-material-negative (CRM^-, Lin et al., 1997). Thus, this model has no endogenous Factor IX species able to compete for biological and physiological activities of infused Factor IX. As assayed by the Factor IX-specific activated partial thromboplastin time (aPTT) assay (an *in vitro* coagulation assay), the clotting time is greatly prolonged. Mice will develop muscle and joint bleeding after fighting with littermates, and Factor IX-knockout mice (in contrast to wild-type mice) will exsanguinate (bleed to death) following a bleeding challenge such as the tail transections described below. Although clotting factors from one species are not always fully functional in plasma of another species, human Factor IX is functional in the murine coagulation system. Human Factor IX corrects murine factor IX-deficient plasma in the *in vitro* aPTT assay (Kung et al., 1998) and corrects the Factor IX-knockout hemophilic phenotype in a bleeding challenge (Kung et al., 1998; Chao et al., 2001). However, infused Factor IX induces an immune response and generation of inhibiting antibodies after about seven days of post-intravenous exposure.

C.4.1. Hemostatic effects of transgenic Factor IX. Factor IX is most often administered to patients after a bleeding event has occurred, which is signaled by pain and stiffening in a joint. Thus, it is

important that the recombinant Factor IX made by the transgenic pig efficiently stop a bleeding event, as well as prophylactically prevent bleeding. We have used a tail transection in a hemophilia B mouse as a model to evaluate the effectiveness of infused transgenic Factor IX to stop a serious bleeding event. Transgenic Factor IX purified according to the method of Lindsay et al. (2004), having a specific activity of 200 IU/mg, was formulated into a buffer similar to BeneFIX®. The model consists of the following sequential steps: (1) anesthetize the mice and collect 30 μl blood to obtain an initial hematocrit reading; (2) transect the tail at time = 0, and then every 30 seconds begin touching the filter paper to the drop of blood forming without disrupting the clot that is forming; (3) at t = 1 minute, inject either transgenic Factor IX or normal saline. Bleeding was halted at 15 minutes if necessary, and six hours post-infusion, a blood sample was taken to determine hematocrit levels. **Figure 7A** shows the typical clotting response for a wild-type mouse with normal hemostasis, a hemophilia B mouse that received no Factor IX treatment, and a hemophilia B mouse that received a 50 IU/kg-bodyweight dose of purified transgenic Factor IX, which is a typical dosage for treating a bleeding event in hemophiliac patients. With this tail transection method, wild-type mice showed a steady decrease in the amount of bleeding, with cessation of bleeding within 13 minutes. The untreated hemophilia B mice receiving no treatment bled profusely for 14 minutes, and the tails were cauterized to prevent exsanguination. The hemophilia B mice receiving 50 IU/kg-bodyweight of transgenic Factor IX showed a steady decrease in bleeding, with cessation of bleeding within about 10 minutes. The decrease in the hematocrit (decrease in the percentage of red blood cell volume, a measure of the total blood loss as a result of the injury) for these mice six hours post-infusion is shown in **Figure 7B**. The wild-type mice having normal hemostasis had an average decrease of 7%±1% (n = 5), untreated hemophilia B mice had an average decrease of 16%±1% (n = 3), and the hemophilia B mice treated with 50 IU/kg of transgenic Factor IX had an average decrease of 3%±1% (n = 5). Thus, the hemostatic effect of intravenous infusion of transgenic Factor IX intravenous into the hemophilia B mouse is equivalent or greater to that of the hemostatic response observed in the normal mouse. We anticipate that transgenic Factor IX produced by cGMP will show similar effects in this and other hemostatic animal models needed prior to Phase I clinical trials.

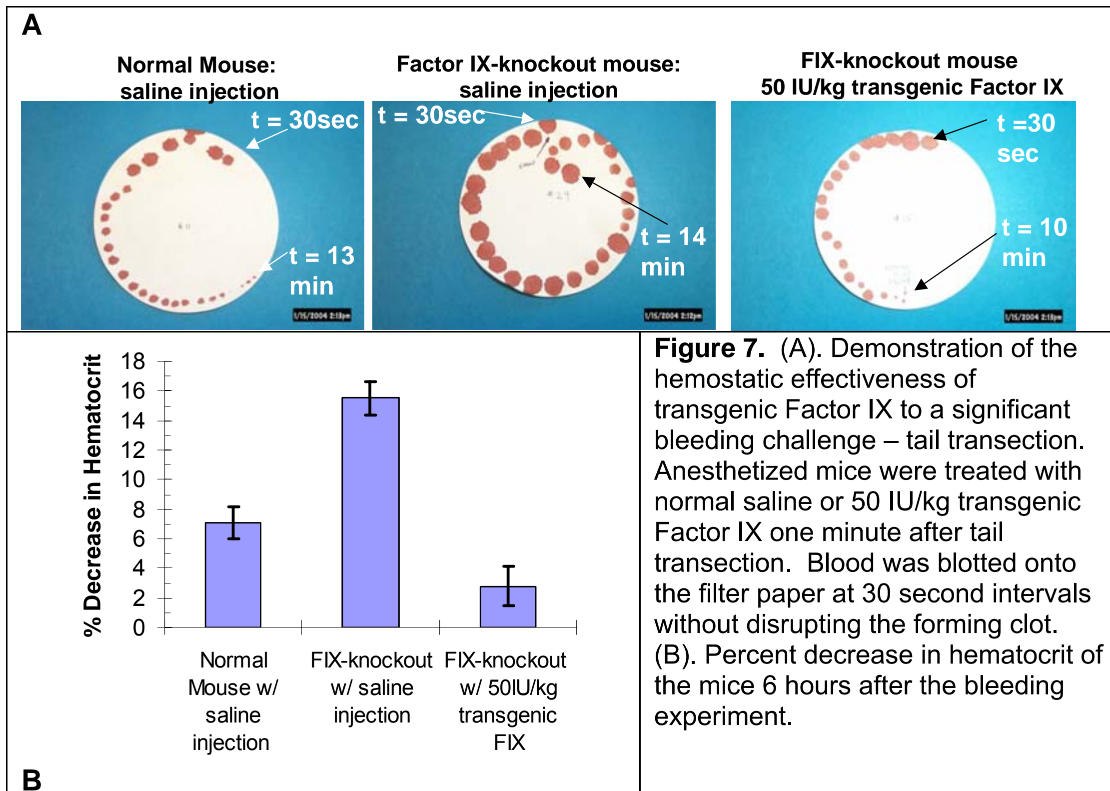


Figure 7. (A). Demonstration of the hemostatic effectiveness of transgenic Factor IX to a significant bleeding challenge – tail transection. Anesthetized mice were treated with normal saline or 50 IU/kg transgenic Factor IX one minute after tail transection. Blood was blotted onto the filter paper at 30 second intervals without disrupting the forming clot. (B). Percent decrease in hematocrit of the mice 6 hours after the bleeding experiment.

C.4.2. Pharmacokinetic behavior of Factor IX and it potential as a prophylactic therapy. In the United States, the most important potential contribution of the abundance of recombinant Factor IX

derived from transgenic pigs is the enabling of prophylactic therapy. As a first step in evaluating the feasibility of prophylactic therapy, we measured the pharmacokinetics of transgenic-derived Factor IX in hemophilia B mice, as assayed by activated partial thromboplastin time. As a reference, it has been recently been reported (Gui et al., 2002) that the pharmacokinetics of human Factor IX in this Factor IX-knock-out mouse are not markedly different from those seen in humans, and it is reasonable to assume that this animal model will allow comparative studies of the activity and kinetics of the transgenic Factor IX versus currently available Factor IX products. In this respect, the Factor IX-knockout mouse model is similar to the hemophilia B dog model, which also demonstrates Factor IX pharmacokinetics similar to those seen in humans (Schaub et al., 1998). Thus, the mouse can be used for studies with large numbers of animals to suggest more optimal approaches for testing in the large animal model.

For all Factor IX products evaluated to date the initial recovery of Factor IX activity immediately after infusion is low, and there is a very large variation among patients. For example, initial recoveries ranging from 21-62% were reported for BeneFIX®, and 31-69% for plasma-derived Mononine® (Morfini, 2003). It is also well known that Factor IX specifically adsorbs to receptors on the vascular endothelium (Heimark and Schwartz, 1983; Stern et al., 1987; Cheung et al., 1996), which likely accounts for the low initial recoveries – the endothelium is an additional reservoir for infused Factor IX. Thus, simple one-compartment pharmacokinetic models are not accurate for Factor IX. "Non-compartment" models have been suggested to be more appropriate for Factor IX pharmacokinetic analysis (Morfini, 2003). These models simply describe the response that is observed and include an inherent assumption of at least one central compartment that is linked to the plasma concentration *vs*. time profile of the drug (Riviere, 1999). The data are analyzed by calculating areas under the curves, using either the trapezoid rule or curve-fitting techniques to integrate the area under the curve (AUC) of the concentration *vs*. time profile, and area under the first moment curve (AUMC). From this information, a *Mean Residence Time* (MRT), the average total length of time that all Factor IX molecules spend in the body, can be calculated (Herman, 2002) as shown in **Table 1**.

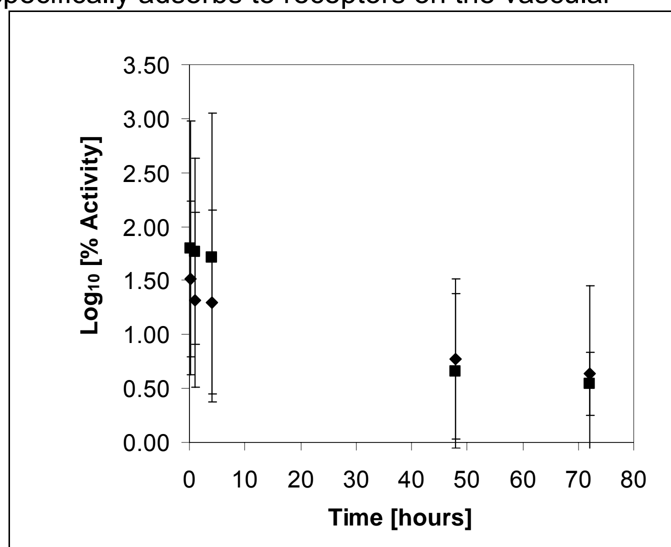


Figure 8. Semi-log plot of Factor IX activity measured in plasma samples taken after intravenous injection of Mononine® (■, n = 8) and transgenic-derived Factor IX (♦, n = 8) in the Factor IX-knockout hemophilia B mouse model. Factor IX activity in collected mouse plasma was measured by the aPTT assay.

We formulated transgenic Factor IX purified by the method of Lindsay et al. (2004) into a buffer similar to that of BeneFIX®. The Factor IX was injected into the hemophilia B mice in replicate treatment groups of 7-8 mice each for plasma-derived Factor IX (Mononine®) and transgenic Factor IX. Factor IX activity and antigen levels recovered at 15 minutes post infusion ranged from 34-65% for plasma-derived Factor IX and 22-26% for the transgenic Factor IX. Shown in **Figure 8** is a semi-log plot of Factor IX activity *vs*. time for one representative set of experiments comparing transgenic Factor IX and Mononine® (% Plasma Activity, where 100% = 1 IU/ml). While plasma-derived material had a higher post-infusion recovery of activity and antigen, the decay of Factor IX activity in the mouse was faster than for the transgenic-derived Factor IX. The Mean Residence Time of transgenic-derived Factor IX was calculated to be 40.0 hours using an arbitrary two-term exponential fit

Table 1. Mean Residence Times (MRT) of Factor IX in Factor IX-knockout mice after intravenous infusion with Mononine® and transgenic-derived Factor IX, calculated from the AUC and AUMC of the data in Figure 8.

Factor IX IV Infusion	MRT [hours]
Mononine® (n = 8)	19.8
Transgenic Factor IX (n = 8)	40.0

of the data (residuals of fit *vs*. data were < 2%). The corresponding Mean Residence Time of Mononine® was 19.8 hours. A similar analysis of BeneFIX® yielded a recovery of about 42% and a Mean Residence Time similar to Mononine®. All Factor IX products were able to sustain about 5% of normal Factor IX activity (0.05 IU/ml) in plasma for 72 hours post-infusion. A level of 5% or more is considered sufficient to render normal blood clotting.

This lower recovery of transgenic Factor IX suggests it is distributed into extra- and circulatory spaces differently than plasma-derived Factor IX or BeneFIX®. We postulate that the long half-life and lower initial recovery in plasma may be an indication that the transgenic recombinant Factor IX accumulates to a greater amount in extra-circulatory compartments that are in equilibrium with plasma. If this is true, the transgenic-derived Factor IX appears to redistribute itself into plasma at a rate that is able to maintain ample amounts of Factor IX needed for normal blood clotting.

C.5. Postprandial, Oral Dosage of Transgenic Factor IX in a Hemophilia B Mouse and Normal Dog Model

We have fed raw transgenic Factor IX milk to Factor IX-knockout mice to broadly and in a facile way confirm that biologically active Factor IX could be transported from gut to plasma. Mice were fed 0.5 ml of transgenic milk after a meal (postprandial), and two methods were used to assess the appearance of biologically active Factor IX in plasma: (1) a combination of ELISA (enzyme linked immunosorbent assay) and aPTT assay of plasma samples taken one hour after post-prandial dosing; (2) bleeding times using a tail transection as a high sensitivity method of *in vivo* hemostasis. It is important to note that no Factor IX activity or Factor IX antigen was detected in mice that were administered Factor IX transgenic milk prior to a meal. Only those mice administered Factor IX transgenic milk after a meal showed levels of Factor IX activity and antigen. Thus, the data below are for postprandial administration.

Table 2. Oral dosage of transgenic Factor IX in raw milk to FIX-knockout mice (n = 4).

Mouse	**Total FIX Antigen by ELISA [μg/ml plasma]**	**aPTT Time [sec]**	**% FIX activity in plasma** (100% = 1 IU/ml)	**Effective concentration of functional transgenic Factor IX [μg/ml plasma]**
1	2.3	73.6	2.7%	0.14
2	7.8	68.4	4.2%	0.21
3	6.4	67.3	4.6%	0.23
4	Not detected	-	-	-

Table 2 shows the distribution between total transgenic Factor IX antigen and biologically active transgenic Factor IX absorbed from the raw milk dosing. In three out of four mice, high levels of total transgenic Factor IX antigen were detected in the mouse plasma. In addition, the Factor IX aPTT activities of the plasma samples for these same three mice were increased. The raw milk fed to these mice contained 100 IU/ml of Factor IX activity, but about 3-4 grams per liter of total Factor IX antigen, as verified by the purification data discussed above. Importantly, the ELISA and aPTT values indicate that relative ratios of total transgenic Factor IX antigen and biologically active transgenic Factor IX appearing in the plasma were the about same as the raw milk fed to them. The results show that 0.2-0.6% of both the total Factor IX antigen and the biologically active Factor IX are transported into the plasma even without the aid of formulation. Thus, there was no selective transport differences between biologically active *vs*. inactive transgenic Factor IX populations, and it demonstrates that transgenic Factor IX can be transported across the gut and into the plasma. The design of this experiment does not yield how much Factor IX was potentially transported to extra-circulatory reservoirs.

In addition to aPTT and ELISA analysis of plasma samples, we evaluated the more sensitive tail bleeding time in the Factor IX-knockout hemophilia type B mouse model. This enabled us to observe

longer-term effects of milk feeding on hemostasis. In this experimental model, normal mice usually stop bleeding after a tail cut within minutes (**Figure 10A**, open squares), whereas hemophilic mice do not stop bleeding and require cauterization of the tail tip after 15 minutes (open circles). When the hemophilia B mice were tested 12 hours after drinking the transgenic milk, the bleeding time was corrected (closed circles). In three mice, we continued the feedings every other day for 1-2 months, and found that the mice did not develop any resistance to the therapy (**Figure 10B**). Intravenous dosages normally results in a 100% incidence of Factor IX-neutralizing antibodies after only 10-12 days in these Factor IX-knockout mice. Importantly, these data indicate two promising potential benefits of oral Factor IX dosing: (1) the ability to maintain hemostasis even with significant bleeding challenges, and (2) the mitigation of the generation of inhibitory antibodies to Factor IX.

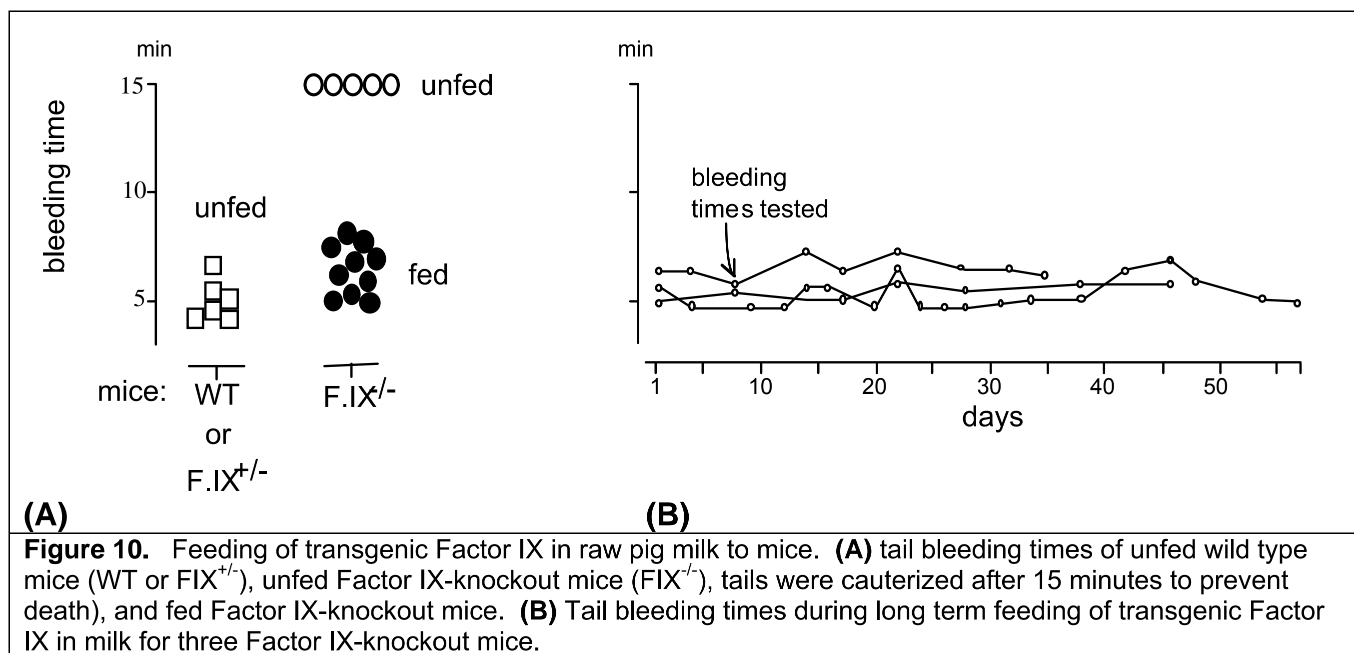


Figure 10. Feeding of transgenic Factor IX in raw pig milk to mice. **(A)** tail bleeding times of unfed wild type mice (WT or $FIX^{+/-}$), unfed Factor IX-knockout mice ($FIX^{-/-}$), tails were cauterized after 15 minutes to prevent death), and fed Factor IX-knockout mice. **(B)** Tail bleeding times during long term feeding of transgenic Factor IX in milk for three Factor IX-knockout mice.

The above mouse experiments using raw Factor IX-containing transgenic pig milk broadly confirm the previous phenomenon of plasma-derived Factor IX transport across the gut in a normal dog that used formulated Factor IX (Horikoshi et al., 1982). We used a similar raw milk feeding experiment in a normal adult dog to further confirm the phenomena of transgenic Factor IX transport from gut to plasma. A 4-year-old, normal mixed-breed dog weighing 20 kg was post-prandially dosed with 50 ml of raw milk containing about 250 IU/ml of transgenic Factor IX activity (about 500 Units of transgenic Factor IX per kilogram of bodyweight). **Figure 11** shows that by two hours, the Factor IX activity in plasma as measured by aPTT was raised by about 20-30%. ELISA values were also raised a similar amount. Based on these results, we calculated that approximately 4% of the administered biologically active transgenic Factor IX was transport into plasma. While this was a preliminary experiment using unformulated transgenic Factor IX, we were able to confirm the general phenomena of biologically active transgenic Factor IX transport from the gut to plasma, as shown by Horikoshi et al. (1982) using liposome-formulated Factor IX. Importantly, we now anticipate that the efficiency of Factor IX appearing in plasma will increase with volume-reduced, highly purified transgenic Factor IX that is optimally formulated for oral dosage. Even with the same low bioavailability shown in the dog experiments, normal blood clotting could be sustained for infants while using a minimal amount of material. For example, consider only the case of infants up to about age 2 having body weights less than 15 kg. It is feasible that normal hemostasis may be achievable in a hemophiliac infant by daily consumption of a purified and formulated Factor IX that is derived from only 50 ml of pig milk. From the point of view of an abundant supply for oral delivery dosage for hemophilia B infants, the Factor IX purified from one day's harvest of milk from one pig (2 liters) could potentially supply 40 oral dosages.

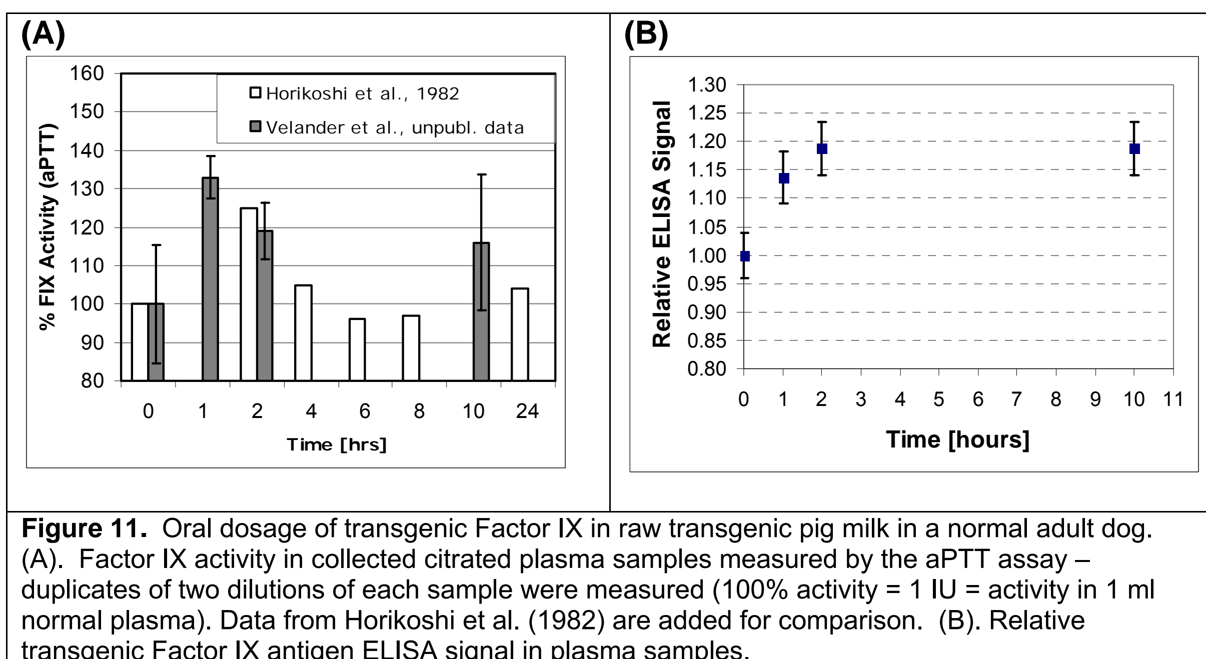


Figure 11. Oral dosage of transgenic Factor IX in raw transgenic pig milk in a normal adult dog. (A). Factor IX activity in collected citrated plasma samples measured by the aPTT assay – duplicates of two dilutions of each sample were measured (100% activity = 1 IU = activity in 1 ml normal plasma). Data from Horikoshi et al. (1982) are added for comparison. (B). Relative transgenic Factor IX antigen ELISA signal in plasma samples.

C.6. Summary of Preliminary Data

The above preliminary animal model data show that the recombinant human factor IX from transgenic pig milk is functional from the perspective of an intravenously administered hemostatic agent and resultant pharmacokinetic behavior. The above studies in the hemophilia B mouse model showed that transgenic Factor IX was kinetically efficient in stopping bleeding after intravenous administration, has an effectively long residence time, and also that it can be transported in a functional form across the gut to plasma at levels that render normal hemostasis. Importantly, the adult hemophilia B mouse model also showed that the oral dosage appeared to confer immune tolerization over a long period of administration as intravenous dosages resulted in a 100% incidence of Factor IX activity neutralizing antibodies after only 10-12 days.

D. Research Design and Methods

The steps that will be required to produce candidate transgenic Factor IX intravenous and oral dosage therapies so that clinical trials can be initiated are as follows:

1. Analytical methods development.
2. Large-scale bioprocess engineering and development.
3. cGMP production.
4. Formulation for intravenous and oral dosage.
5. Comprehensive testing in hemophilia B mouse and dog models.

The minimum goal of this work will be a transgenic Factor IX intravenous therapy that is ready to enter clinical trials. The experience we will gain from achieving this objective will be used to develop an oral dosage transgenic Factor IX. Preparation for cGMP production, IND filing, clinical trials, and successful FDA approval requires thorough documentation at all levels. Thus, our program emphasizes leading-edge document management technology and specialized staff for its implementation. This has also been a key consideration in other University of Nebraska-Lincoln Biological Process Development Facility funded projects (NIAID and US Army) whose goal is the generation of Phase I and II clinical materials. We also have specialized and centralized program managerial staff to coordinate and document activities of each site relative to monthly milestones.

D.1. Specific Aim # 1. Process engineer and scale-up the recovery and purification of transgenic recombinant human Factor IX

The *justification* for Specific Aim #1 is that a large-scale cGMP purification process and a matrix of analytical methods are needed to produce transgenic Factor IX suitable for clinical trials. A further advantage is that the material used for preclinical studies in dog models for both intravenous or oral dosage applications will be more reflective of the anticipated results in humans. Furthermore, the availability of hemophilia B dogs limits the number of permutations of Factor IX formulations that can be studied. Thus, while mice can be used with Factor IX derived from intermediate stages of development, the most reliable and highly characterized transgenic Factor IX formulations should be used in dogs. cGMP material represents the highest degree of reliability. The *objective* of this specific aim is to complete all aspects of process development, scale-up, and cGMP manufacturing for production of candidate populations of recombinant Factor IX for evaluation in hemophiliac animal models.

There are five critical steps to successful implementation of a cGMP production process. First, ProGenetics LLC will provide a reliable supply of milk from transgenic pigs producing up to 4 grams transgenic Factor IX/liter (100-1000 U/ml). Second, analytical methods and in-process testing must be established to characterize the milk and all intermediates resulting from process purification. Third, standard operating procedures (SOPs) must be written and transferred to a quality control laboratory to ensure a consistent and reproducible analysis. All of these methods will eventually be validated prior to cGMP manufacturing for clinical trials, but for the work outlined in this proposal, the methods will only be qualified. Fourth, the purification process and formulation must be developed to ensure a consistent process that produces a candidate transgenic Factor IX product. During this process engineering phase, we will confirm that candidate products have desired hemostatic and pharmacokinetic properties in the hemophilia B mouse model. Fifth, the Biological Process Development Facility cGMP staff will establish standardized production batch records, run at the pilot-scale, finalize the production batch records, and finally run the cGMP process to produce transgenic Factor IX suitable for preclinical studies in hemophilia B dogs and other animal models used to document safety characteristics prior to Phase I clinical trials.

In summary, the *rationale* for *Specific Aim #1* is that successful completion of the proposed research will produce two necessary Factor IX products: intermediate transgenic Factor IX that will guide process development that will result in a Factor IX candidate product with desirable hemostatic and pharmacokinetic properties; and a final transgenic Factor IX product for evaluation in hemophilia B dogs.

D.1.1. Transgenic pig milk. Starting on Day 5 of lactation, piglets will be separated from their mother for a period of one hour. At the end of that period, the mother will be led into a milking pen and 60 units of oxytocin (Vedco, 3ml) will be administered intramuscularly to the animal. The udder will be washed and 5 to 8 minutes after administering oxytocin the animal will be milked by hand or by machine for approximately 20 minutes. After milking the animal, it will be released and united with its offspring. Milkings will occur up to three times a day. The animal will have free access to food and water during the procedure.

Milk will be shipped frozen according to USDA regulation for transgenic animal-derived materials to the University of Nebraska-Lincoln. Fresh-frozen daily milk samples from five animals spanning an entire lactation of 50 days will be sent. Aliquots of 10 x 1 ml, 10 x 10 ml, and 1 x 250 ml of each sample will be shipped. An identical set of milk samples will be retained at ProGenetics stored at –80°C. Nontransgenic milk samples will be shipped and used as controls. Pre-screening of transgenic milk samples before they are admitted to process development or production will use routine assays we already have in place:

1. Measurement of transgenic Factor IX concentrations by ELISA.
2. Factor IX activity levels by the aPTT assay.
3. Molecular fingerprint by reduced western blot so that milk samples with high levels of activated Factor IX (FIXaβ) are excluded.

D.1.2. Analytical methods development. The justification for our methods development is based upon the anticipated requirements for an Investigational New Drug filing needed for clinical trials of transgenic Factor IX. These methods are largely based upon the precedent already set by the FDA approval of the only recombinant Factor IX product in the United States (BeneFIX®) and the only plasma-derived products (Mononine® and AlphaNine®SD). The Target Product Profile for transgenic Factor IX is given in **Table 3**. The objective will be to quantify the properties of cGMP-produced transgenic Factor IX products and obtain an abundant product that is equivalent to or better than FDA-approved Factor IX products. As the project progresses through the Process Development stage (beginning in Year 2) and the cGMP Manufacturing stage (through Year 5), these properties in the Target Product Profile will be quantified and refined. This will be an important task of the Lead Investigators and the Scientific Steering Group. In subsequent renewal proposals and prior to clinical trials, additional specifications regarding product quality, formulation, dosage, and pharmacokinetics will be implemented.

Table 3. Target Product Profile for Transgenic Factor IX.

Property	Specification and Justification
Biological activity	≥ 150 IU/mg; Similar to BeneFIX® and plasma Factor IX products
γ-carboxyglutamate (Gla) content	≥ 6 moles Gla/mole Factor IX; ≥ 6 Gla's are required for biological activity
Carbohydrate content	complex N-linked glycans with terminal sialic acid; Similar to BeneFIX® and plasma Factor IX
Ser-phosphorylation content	≥ 1 mole phosphoserine/mole Factor IX; Better than BeneFIX®, similar to plasma Factor IX
Molecular weight	Factor IX monomer; Similar to BeneFIX® and plasma Factor IX
Tyr-sulfation content	≥15%; Better than BeneFIX®, similar to plasma Factor IX
Activated Factor IX	To be determined; upper limit will be defined by thrombogenicity in large animal model
Propeptide content	To be determined; Similar to BeneFIX® and plasma Factor IX
Contaminants – host proteins	To be determined; Similar to BeneFIX® and plasma Factor IX
Thrombogenicity – host coagulation proteins	To be determined; Similar to BeneFIX® and plasma Factor IX
Contaminants – endotoxins	To be determined; Similar to BeneFIX® and plasma Factor IX
Contaminants – host nucleic acids*	To be determined; Similar to BeneFIX® and plasma Factor IX
Viral pathogens*	To be determined; Similar to BeneFIX® and plasma Factor IX
Pharmacokinetics	To be determined for animal models; Similar to BeneFIX® and plasma Factor IX
Long-term stability*	Two-year shelf life for lyophilized product

* Quantitatively determining these product specifications will not be part of this proposed project

The methods that will be used in this project to evaluate our transgenic Factor IX products relative to these specifications are listed in **Table 4**. We have prioritized product characteristics that are directly related to producing a consistent and high quality transgenic Factor IX product that is suitable for evaluation in the hemophilia B mouse and dog models. We recognize that before clinical trials are initiated, additional methods will need to be put in place to verify that higher standards will be met. Results obtained from these assays will provide important information about the following issues:

1. Efficacy of the transgenic Factor IX as a procoagulant enzyme
2. Safety of the molecule with regard to
    a. sterility and pyrogenicity
    b. thrombogenicity (*in vitro* assays only)
    c. host protein contamination
3. Stability of the transgenic Factor IX product candidates (in the timeframe of the project)
4. Structure of post-translational modification features of transgenic Factor IX that determine biological activity and pharmacokinetic properties.

Table 4. Analytical Methods that will be developed and used to characterize transgenic Factor IX for this project and support activities in Process Development, Quality Control, and cGMP Manufacturing.

Purpose	Assays
Factor IX Characterization (structure and function)	▪ aPTT activity (Barrow et al., 1959; Lindsay et al., 2004) ▪ Chromogenic activity (Prasa and Sturzebecher, 1998) ▪ Propeptide ELISA (Bristol et al., 1994) ▪ Carbohydrate analysis (Chen et al., 1998) ▪ Phosphorylation (Schulenberg et al., 2003; Bennett et al., 2002) ▪ Tyr-sulfation (Severs et al., 1999) ▪ γ-carboxylglutamate content (Ware et al., 1989; Bond et al., 1998) ▪ N-terminal sequencing (Bond et al., 1998) - at University of Nebraska Medical Center Protein Structure Core Facility ▪ Amino acid analysis – at University of Nebraska Medical Center Protein Structure Core Facility ▪ Peptide map – oxidation, deamidation, proteolysis (Bond et al., 1998)
In-Process Assays (to confirm product purity and biological activity of intermediate products)	▪ aPTT activity (Barrow et al., 1959; Lindsay et al., 2004) ▪ Chromogenic activity (Prasa and Sturzebecher, 1998) ▪ Factor IX concentration (HPLC) (Lindsay et al., 2004) ▪ Molecular weight (SDS PAGE; Size exclusion chromatography) (Bond et al., 1998)
Process Characterization Assays	▪ Host protein – Albumin (Van Cott et al., 2001) ▪ Host protein – IgG (Van Cott et al., 2001) ▪ Host protein – IgA (Van Cott et al., 2001) ▪ Host protein – IgM (Van Cott et al., 2001) ▪ Thrombogenicity – porcine coagulation factors by factor-specific *in vitro* coagulation assays. ▪ Endotoxins – LAL assay at Univ. Nebraska-Lincoln BPDF ▪ Hemophilia B mouse model bioassay – at UNC-Chapel Hill
Product Release Assays (includes product stability assays)	▪ aPTT activity (Barrow et al., 1959; Lindsay et al., 2004) ▪ Chromogenic activity (Prasa and Sturzebecher, 1998) ▪ Factor IX concentration (HPLC) (Lindsay et al., 2004) ▪ Molecular weight (SDS PAGE; Size exclusion chromatography) (Bond et al., 1998) ▪ N-terminal sequencing (Bond et al., 1998) ▪ Amino acid analysis ▪ Peptide map (Bond et al., 1998) ▪ Host proteins (Van Cott et al., 2001) ▪ Thrombogenicity – porcine coagulation factors by factor-specific *in vitro* coagulation assays. ▪ Endotoxins - LAL assay at Univ. Nebraska-Lincoln BPDF ▪ Bioburden - at Univ. Nebraska-Lincoln BPDF
Raw Material Release Assay for cGMP production	▪ IR, LAL, bioburden, pH, conductivity, HPLC - at Univ. Nebraska-Lincoln BPDF

BeneFIX®, AlphaNine®SD, and Mononine® will be used as standards for all Factor IX structure/function assays. Nontransgenic milk samples will be used as controls where warranted. The investigators and staff at the University of Nebraska-Lincoln Biological Process Development Facility will focus heavily on method development in Years 1-2 of this project. Standard Operating Procedures (SOPs) will be developed and transferred to the Quality Control group, and these methods will be used to support efforts in Process Development, Formulation, and cGMP Manufacturing.

D.1.3. Process development – bioseparations engineering and scale-up. The justification for a pilot-scale purification process is that a candidate material must be reliably produced within a defined product specification, in a quantity sufficient for preclinical evaluation in hemophilia B dog models, and a quality to later supply subsequent clinical trials. The advantage of using cGMP material is having a defined product that can reliably be correlated with *in vivo* behavior and that will be less subject to artifacts that can occur in products made at a less regulated lab bench environment. This advantage speeds the leap to supplying material for clinical trials.

D.1.3.1. Initial recovery of transgenic Factor IX. The goal of the recovery step is to remove milk fat and somatic cells, producing a "skim milk" stream that maximizes the recovery of transgenic Factor IX. The centrifuge, i.e., cream separator, is used in the dairy industry to produce skim milk. Another technology that has potential for this separation is crossflow membrane filtration. The advantage of crossflow membrane filtration is that it can facilitate the separation of other milk proteins from transgenic Factor IX, which could dramatically impact the first capture column, since skim milk contains up to 60 g/L of "contaminating" proteins. Parameters that will be evaluated are membrane pore size, pH, ionic strength, and linear velocity, which can affect the membrane gel layer and separation efficiency. Given the right conditions, it is possible to separate proteins based on size and charge using crossflow membrane filtration. Precipitation will also be evaluated to reduce the level of milk proteins. A successful recovery method will be determined by the impact on the binding capacity of the first capture column. Centrifugation will be the "bench-mark" to which the other methods will be compared.

D.1.3.2. Capture step. The goal of the capture step is to volume-reduce and achieve some level of purification. The method should be robust and simple with elution of the desired transgenic Factor IX-containing product with step changes in buffer conditions. As discussed above, heparin affinity chromatography has proven to be an effective capture step because it selectively and quantitatively adsorbs transgenic Factor IX. However, heparin columns are expensive and potentially leach porcine heparin as a contaminant into the product. Thus, we will investigate mimetics of heparin affinity matrices such as dextran sulfate and sulfated cellulose. Anion exchange chromatography can also be used to quantitatively bind transgenic Factor IX, but nonspecific, strong adsorption of milk proteins such as caseins occurs. In particular, beta-casein is very difficult to partition away from transgenic Factor IX. Thus, heparin mimetics will be prioritized.

D.1.3.3. Viral reduction. The goal of viral reduction processing steps is to achieve log decreases of the viral activity in specific classes of viruses with minimal loss of product activity. Typically, viral reduction steps are applied after the capture step to benefit from volume-reduction. These technologies differ in the class of viral pathogen that is inactivated. Model viruses of specific classes are typically spiked into the feedstream and the amount of reduction measured. Typical technologies for viral reduction used in commercial available therapeutics are solvent/detergent for lipid envelope viruses, nanofiltration for non-lipid envelope viruses, and heat treatment for all viruses. Chromatographic processing can also provide a barrier to pathogens (Hay, 2002). As a barrier to lipid envelope viruses, we will adapt a solvent/detergent reduction step using tri-n-butylphosphate (TNBP) and Triton X-100 (Horowitz et al., 1992). In addition, while ProGenetics' transgenic pig herd is of free porcine parvovirus, the transgenic Factor IX product from the polishing step will treated by nanofiltration to provide an additional barrier to non-lipid-coated viruses such as parvovirus (Van Holten et al., 2002). In this work, we will determine the stability of transgenic Factor IX activity in response to

solvent/detergent treatment and nanofiltration. The validation of viral reduction will be the subject of a subsequent grant proposal.

D.1.3.4. Purification and polishing steps. The goal of the purification and polishing steps is to produce a final transgenic Factor IX bulk product having the purity, biological activity, and pharmacokinetic properties given in the bulk product specifications. Important contaminants that must be removed include inappropriate proteases such as activated transgenic Factor IX, porcine prothrombin, porcine Factor VII, and porcine Factor X. As mentioned earlier, the purification and polishing of transgenic Factor IX produced by Lindsay et al. (2004) using high resolution anion exchange chromatography yielded a transgenic Factor IX product with a 40-hour Mean Residence Time in hemophilia B mice, nearly double that of BeneFIX® and Mononine® in the same model. Because this polishing step is scalable, we will use this technique as a starting point. The analytical methods described above will be critical to evaluating the effectiveness of the polishing step, especially with respect to removal of thrombogenic contaminants.

Process development at the bench-scale will be conducted with 7.8 to 100 ml columns packed with matrices suitable for process-scale using Perseptive Biosystems BioCad process chromatography systems, which are capable of 100 ml/min flow rates. Once a process has been finalized at the 100 ml column-scale, it will be scaled to the 1 to 2 L column scale using pilot-scale chromatography equipment in the BPDF Purification Development Laboratory. For its scale-up efforts, the Purification Development Lab has an Amicon K-prime 40 chromatography system (500 ml/min) and a North Carolina SRT chromatography system capable of 1000 ml/min. A North Carolina SRT Model 50 crossflow membrane filtration system capable of running 2 to 3 m^2 of ultrafiltration membrane is capable of processing 5 to 20 liters of sample. The process will be run three successive times at the pilot-scale, with all of the supporting analytical testing to confirm the process and provide all of the information necessary for technology transfer to cGMP manufacturing of the final bulk product.

The BPDF will also be supporting formulation activities at Colorado State University (described in Specific Aims #2 & #3). It will be responsibility of the BPDF to produce transgenic Factor IX for Colorado State as needed during the grant for formulation and lyophilization studies. Both non-cGMP and cGMP sterile bulk will be produced at UNL, and lyophilized final Drug Substance will be produced at Colorado State in a fashion that guarantees material suitable for preclinical trials in hemophilia B dogs.

D.1.4. Factor IX-knockout hemophilia B mouse bioassay for process development. The Factor IX-knockout mouse is an excellent model of the human hemophilia B phenotype and will be used for evaluating transgenic Factor IX candidate preparations made during Process Development. As reviewed above, the Factor IX-knockout mouse produces no mRNA transcript, has no circulating factor IX antigen, and has a bleeding phenotype. Also, human Factor IX is active in the murine coagulation system, and the pharmacokinetics of infused human Factor IX in this mouse model are similar to the hemophilia B dog model and humans (Gui et al., 2002).

D.1.4.1. Infusing and sampling*.* Samples of transgenic Factor IX from Process Development will be formulated in a buffer similar to BeneFIX® (or in a more optimal formulation as the formulation studies progress) and shipped to Prof. Monahan. Commercially available Factor IX (Mononine®, BeneFIX®) and transgenic Factor IX preparations will be injected intravenously (external jugular; 5-7 mice per group). On the day prior to injection, mice will be tagged and weighed, and a plasma sample will be taken to establish baseline Factor IX clotting activity. Mice will be anesthetized with intraperitoneal avertin prior to injection, and the Factor IX sample diluted into a total volume of 100 μl using sterile normal saline. After injection, the injection site will be sutured closed. Plasma samples will be taken at 15 min, 1 hour, 4 hours, 24 hours, 48 hours, and 72 hours from retroorbital venous plexus into citrated collection tubes.

D.1.4.2. Factor IX activity and antigen assays. Samples will be analyzed at UNC-Chapel Hill by aPTT (Start4 Coagulometer) for Factor IX activity and ELISA for Factor IX antigen levels. Thirty minutes after injection, the level of anesthesia will be checked, and avertin dosing will be repeated if it is necessary to keep the animal sedated past 1-hour timepoint. For each subsequent sample, collection will alternate between eyes for retroorbital venous plexus to minimize any effect of partial activation of coagulation *via* tissue factor exposure. The mice will be euthanized after the 72-hour timepoint. The data will be plotted and analyzed to determine the pharmacokinetic properties of the transgenic Factor IX – e.g., area under the curve, Mean Residence Time, volume of distribution, and half-life. The pharmacokinetic parameters will be determined using the commercially available software package (e.g., PK Solutions or PCNONLIN). These results will then guide decisions by the Process Development team, whose goal is to purify transgenic Factor IX having optimal pharmacokinetics.

D.1.5. cGMP manufacturing and quality assurance. The process development information from the pilot-scale studies completed in the Purification Development Laboratory of the Biological Process Development Facility will be used to write the Production Batch Records. This activity is a three-way process among the purification development staff, the cGMP production staff, and the quality assurance staff. Prior to this phase in the project, all of the analytical methods required for in-process testing and release testing are expected to have been completed and reference to these methods will be made in the Production Batch Records. Production Batch Records include all aspects of the production process, from facility change control (facility and equipment cleaning and elastomer change out prior to the cGMP production), specifying all raw materials, buffer preparation, and finally the process to produce Final Bulk Product.

Once the Production Batch Records are completed and signed-off from the Quality Assurance group, they will be issued to the cGMP staff. It is expected that changes will be made to the Production Batch Records prior to the final cGMP run. To this end, one engineering run will be completed using the Production Batch Records to ensure that the batch records accurately reflect the process using the cGMP pilot plant equipment. The typical engineering run will be up to 10 liters of milk in scale for the purposes of hemophilia B dog studies. Changes made to the Production Batch Records during the first engineering run will be incorporated and reissued prior to the second engineering run. Based on the three non-cGMP runs in the Purification Development Laboratory, it is expected that the new batch records will be in sufficient shape to support a successful second engineering run that will produce Factor IX suitable for preclinical studies. Equally important is that product from the second engineering run will meet final product specifications that are decided upon in Section D.1.2.

The product from the first engineering run will be used to finalize the formulation batch records and to “shake down” the lyophilization process. The material from the second engineering run will be used to produce the preclinical cGMP Final Drug Substance that will be used for both the IV and oral dosage canine studies. Once the production phase begins and product candidates have been chosen, we will initiate stability studies of sterile bulk and formulated candidate intravenous products. The sterile bulk stability will serve as a source material for the final formulation of oral dosage candidate formulations. The assays used will be aimed at determining if aggregation, deglycosylation, oxidation, and proteolysis are occurring for sterile bulk and formulated, vialed candidate products (Table 4).

D.1.6. Electronic data capture and technology transfer. The Biological Process Development Facility uses CyberLAB®, an electronic data management system to collect, organize, warehouse, index, and safely archive all structured and unstructured electronic records from raw data and laboratory reports into compliance records, putting the information the BPDF needs to improve operations in one, convenient, easily accessible location. CyberLAB® provides a secure, Part 11-compliant, centralized electronic library for any and all electronic data files of any size. Not only can machine readable instrument data files be stored, but also images, multimedia files, presentations, human readable word processing and Adobe PDF documents, spreadsheets, and hundreds of other formats. CyberLAB® automatically extracts searchable metadata from each of the files using

CyberLAB® SmartFilters. Using a Web-based Virtual Personal Network (VPN), all members of the grant, including administrative personnel from the NIH, will be able to search for any records of interest using an integrated Quick or Advanced query and view the files in the browser without the need for data generating applications for most file types. The BPDF will be able search and retrieve vital information quickly and easily and spend more time using information than searching for it.

All of the work proposed has the primary objective of transitioning transgenic Factor IX into cGMP manufacturing and clinical trials. All process development information is a critical component of the life cycle of the product and is subject to FDA inspection. Having a single repository of this information in a validated electronic format that is completely searchable will have a significant impact on this project and in the future as transgenic Factor IX becomes a commercial product.

D.2. Specific Aim #2. Characterize and formulate transgenic recombinant human Factor IX for intravenous dosage, and evaluate in a hemophilia B dog model

The *justification* for Specific Aim #2 is that the hemophilia B dog model is an established preclinical evaluation needed prior to Phase I clinical trials. The *objective* of this Specific Aim is to evaluate the pharmacokinetics of a cGMP-produced transgenic Factor IX product formulation. The cGMP-produced transgenic Factor IX will be formulated for intravenous delivery using existing information known for BeneFIX® and administered to hemophilia B dogs. Our *rationale* for Specific Aim #2 is based upon past experience with plasma-derived Factor IX and BeneFIX®; an intravenous product that is efficacious in hemophilia B dogs is expected to be efficacious in humans (Brinkhous et al., 1996).

D.2.1. Intravenous formulation. The *justification* for this task is that freeze-dried protein formulations display the most desirable long-term physical and chemical stability for intravenous formulations. In our hemophilia B mouse experiments reported in Section C.4, we used the BeneFIX® formulation conditions reported in Bush et al. (1998) for storing and shipping frozen liquid transgenic Factor IX samples. The *objective* here is to formulate transgenic Factor IX in a similar fashion to BeneFIX® for a *lyophilized* product. The excipients and Factor IX concentrations that were used to minimize aggregation and obtain the best cake formation for BeneFIX® are as follows:

- 500 and 1000 IU vials: 10 mM l-histidine, 260 mM glycine, 1% sucrose, 0.005% polysorbate 80, pH 6.8.
- 250 IU vials: 5 mM l-histidine, 130 mM glycine, 0.5% sucrose, 0.0025% polysorbate 80, pH 6.8.

The formulation of plasma-derived Factor IX products is also available in their product labels, and they contain similar constituents. Briefly, these formulations are designed to prevent protein unfolding, protein aggregation, and protein degradation. Excipients, especially sugars, replace the water molecules during drying (Carpenter et al., 1997, 2002).

Formulation experiments will begin in Year 2 as products from Process Development are made available. Our initial intravenous formulation will mimic the BeneFIX® formulation, and the following properties of formulated and lyophilized transgenic Factor IX will be measured:

1. Biological activity of reconstituted Factor IX – determined by aPTT after reconstitution.
2. Native protein structure – determined by Fourier transform infrared spectroscopy and circular dichroism.
3. Glass transition temperature of the lyophilized cake – determined by Differential Scanning Calorimetry (a higher glass transition temperature reduces the rate of diffusion controlled reactions such as unfolding and aggregation).
4. Proteolytic damage of reconstituted transgenic Factor IX – by SDS-PAGE
5. Aggregation of reconstituted transgenic Factor IX – by size exclusion chromatography and dynamic light scattering.

Chemical degradation, such as deamidation and oxidation, is also possible and will monitored by peptide mapping and LC-MS of reconstituted transgenic Factor IX. If deamidation of transgenic Factor IX is problematic, it can be significantly retarded by choice of pH, using minimal buffer concentrations, and restricting mobility (have a high glass transition temperature). Although these steps, along with proper lyophilization, will slow most all deamidation processes, it can still proceed in some proteins, especially if they are stored near the glass transition temperature (Carpenter and Chang, 1996). Oxidation of transgenic Factor IX is likely to be more problematic. Methionine oxidation can be minimized by sealing vials under nitrogen and/or using 'sacrificial additives' (e.g., free methionine) to compete with protein residues for reactive oxygen species.

D.2.2. Hemophilia B dogs at UNC-Chapel Hill – IV delivery and pharmacokinetics. The *justification* for this study is that pharmacokinetic studies in hemophilia B dogs are a necessary preclinical model as recognized by the FDA in approved Factor IX products (Mononine®, Alphanine®SD, BeneFIX®), where all of these products were evaluated in hemophilia B dog models prior to human clinical trials. The preclinical evaluation of BeneFIX® was studied in the same lineage of hemophilia B dogs at UNC-Chapel Hill (Brinkhous et al., 1996), and we will perform a similarly designed study. These dogs do not produce Factor IX or cross-reactive material that could compete with exogenously administered Factor IX. However, these dogs will produce inhibiting antibodies to Factor IX within 14 days after IV administration. While IV administration of human Factor IX products acutely corrects coagulopathy in these hemophilia B dogs, the inhibitors occurring in the majority of exposed animals make it impossible to conduct long-term studies (>14 days) that determine the effects of therapy on bleeding frequency. Subsequent proposal applications will focus on studying the pharmacokinetics of transgenic Factor IX in pretolerized hemophilia B dogs (Russell et al., 2003).

D.2.2.1. Short-term cross-over study. A short-term cross-over study is planned in Year 4 to measure the pharmacokinetics of cGMP-produced transgenic Factor IX product. Three hemophilic dogs in a cross-over design is sufficient for a given dose in a non-tolerized dog (Brinkhous et al., 1996). In the first two dogs, plasma-derived Factor IX (Mononine®) will be given on Day 1 and transgenic Factor IX will be given on Day 3 (i.e., 72 hours after infusing Mononine®). For the third dog, transgenic Factor IX will be administered on Day 1 and Mononine® on Day 3. These studies will provide pharmacokinetic data (recovery and half-life) that normalize for individual dog responses to exogenous Factor IX, because each dog serves as its own control. The goal is to give 100 IU/kg based on activity determined by using canine deficient plasma. It is anticipated that both commercial products will be infused according to the commercial manufacturers' recommendations on the package inserts in terms of time and reconstitution, but dosing will be done based on activity in canine plasma.

D.2.2.2. 14-day infusion study. In Year, 5 we will perform a longer-term study with daily intravenous administration of transgenic Factor IX, as was also performed by Brinkhous et al. (1996) for BeneFIX®. This *objective* of this study will be to evaluate the pharmacokinetics of transgenic Factor IX in a dosing regimen that models prophylaxis – it was found that the initial recovery of BeneFIX® in a daily dosing regimen increased by more than double that of a single bolus. Doses of 50 IU/kg for up to 14 consecutive days will be administered to three dogs. Based on previous experience with BeneFIX®, we expect that there will be no immune response or reactions during the first 9 days. Treatment will be halted if the dogs develop reactions.

D.2.2.3. Sampling. Platelet-poor plasma will be prepared by double centrifugation of blood anticoagulated with 3.2% sodium citrate. Plasma samples will be assayed immediately or frozen at negative 80°C until assayed. Pools of normal dog plasma and plasma from four hemophilia B dogs that have not been treated with any plasma products will be prepared as controls. The assays will include the whole blood clotting time (WBCT), aPTT, and Factor IX activity and antigen assays. A sufficient number of samples will be drawn to be able to calculate pharmacokinetic parameters including the maximal plasma concentration, half-life, area under the curve, bioavailability, and volume of distribution. If these Factor IX products have a longer half-life, sampling will be modified to be able to detect this

outcome and allow washout before cross-over. It is possible that the transgenic Factor IX will prolong the half-life beyond the stated sampling interval; additional samples will be taken twice daily until the whole blood clotting time becomes prolonged. Serum samples will be prepared to assess the degree the immune response to the Factor IX products and for clinical chemistry analyses (liver enzymes, serum electrolytes, glucose, creatine, amylase, BUN).

Except for the WBCT, all assays will be done at the same time after the infusion and sampling is completed. To detect any systemic toxicity of the infusates, serum for clinical chemistry will be drawn at baseline and daily during the infusion period. The clinical chemistry analyses will be performed every other day by automated methods by Antech, a commercial veterinarian clinical chemistry laboratory. If any toxicity is detected, then all samples surrounding the days in question will be processed. All dogs will be maintained for at least 30 days. Samples will be drawn at days 7, 14, 21, and 28 to assay anti-Factor IX inhibitory antibodies. After the study is completed, the dogs will either be returned to the colony or euthanized.

D.2.2.4. Inhibitory antibody assays. The possibility of an anti-human coagulation factor antibody developing by Day 10 is acknowledged in this study design. The development of this inhibitory antibody may mask persistent activity. All bleeder animals will be tested for pre-existing anti-Factor IX neutralizing antibodies before being assigned to protocols. Factor IX antibody assays will be performed using two procedures, the Bethesda inhibitor assay (Kasper et al., 1975) and a direct binding ELISA with immobilized human Factor IX and enzyme-coupled anti-immunoglobulin reagents (Russell et al., 2003). For the Bethesda inhibitor assay, a patient's plasma with a residual Factor IX activity of 50% of the normal control is defined as one "Bethesda unit" of inhibitor per ml. For ELISA, values will be determined as described (Russell et al., 2003). All of these assays are currently available at the Frances Owen Blood Research Lab (FOBRL), where the hemophilia B dogs are produced, maintained, and given the experimental Factor IX preparations.

D.2.2.5. Thrombogenic potential and coagulation assays. Potential procoagulant effects including disseminated intravascular coagulation will be assessed by measuring platelet counts at baseline, 4, 8, 24, 48, and 72 hours post infusion. The prothrombin time, fibrinogen, fibrin split products, and prothrombin fragment 1+2 levels will be measured at baseline, 4, 8, 24, 48, and 72 hours by Esoterix, Aurora, Colorado.

Correction of the whole blood clotting times (WBCT) has now been shown to correlate with reduced bleeding in the hemophilic dogs (Russell et al., 2003). The WBCT will be assayed at baseline, 1 hour, 8 hours, 24 hours, and daily until prolonged to a significant length (i.e., at least doubled from the nadir following treatment). More frequent assaying will be attempted when the WBCT begins to prolong.

Factor IX coagulant activities will be determined by a modified one-stage partial thromboplastin time using canine Factor IX-deficient substrate plasma assay (Barrow et al., 1959). Normal canine reference plasma consists of pools from 5-10 normal dogs from our inbred colony. The test sample will be diluted several fold and compared to the same dilutions for a normal curve. The results will be reported as a percent of normal. Activated Partial thromboplastin times (aPTT) will be determined in the ST4 coagulation instrument (Diagnostica Stago, Asnsieres, France) or the Multiple Discrete Analyzer (MDA) 180 (bioMerieux, Durham, NC) that has the capacity to process rapidly a large number of samples. Whether the aPTTs are determined on the ST4 coagulation instrument or the bioMerieux MDA 180 (previously Orgenon Teknika), the controls and reagents will be of the same type. For the aPTT test, mixtures will consist of equal portions of partial thromboplastin (Automated aPTT, bioMerieux), 0.025 M $CaCl_2$, and citrated test plasma (Langdell et al., 1953).

D.2.2.6. Factor IX antigen assays. The ELISA testing for canine Factor IX antigen has been performed using commercially available reagents (Herzog et al., 2000). The ELISA for transgenic

Factor IX has been developed by Dr. Van Cott and has a very low cross-reactivity with canine Factor IX in normal dog plasma.

D.2.2.7. Pharmacokinetic analyses. The pharmacokinetic parameters will be analyzed using the commercially available software package (e.g., PK Solutions or PCNONLIN).

D.3. Specific Aim #3. Develop an oral dosage form of transgenic Factor IX, and evaluate in hemophilia B mice and dog models

The *justification* for developing oral dosage forms of transgenic Factor IX is to use defined transgenic Factor IX material to evaluate the bioavailability in mouse and dog models. Our approach is to formulate highly purified transgenic Factor IX in a pharmaceutically acceptable carrier for oral dosage, and to evaluate these formulations in a sequence of a normal mouse model, the hemophilia B mouse model, a normal dog model, and finally the hemophilia B dog model. We expect that bioavailabilities obtained with defined product material in hemophilia B mice and dog models will lead to more advanced animal models, such as primates, and eventually to human clinical trials.

D.3.1. Oral dosage formulation. In anticipation of an oral dosage of transgenic Factor IX that will be first targeted for treating infants, we will focus on lyophilized formulations that are reconstituted as liquids for oral administration. In future studies, gel capsule formulations that are more appropriate for adults will be investigated. The oral dosage formulation of transgenic Factor IX will be guided by our prior results in hemophilia B mice and normal dogs and by previously published results of liposomal formulations of plasma-derived Factor IX and Factor VIII (Hemker et al., 1980; Horikoshi et al., 1982). We expect it is likely that the combination of protease inhibitors and encapsulation of Factor IX in liposomes increased the bioavailability obtained by Horikoshi et al. (1982). While our preliminary study used raw transgenic milk as a delivery vehicle, it is clear that the simple presence of a lipophilic mixture is insufficient to obtain the bioavailabilities found by Horikoshi and Hemker. Thus, a specific formulation process to encapsulate transgenic Factor IX, along with addition of inexpensive protease inhibitors like soybean trypsin inhibitor, should increase the bioavailability above that found with raw milk. In addition, transgenic Factor IX was only detected in the plasma of mice and dogs if the milk was fed after a meal. Thus, it is likely that titration of gastric pH by the buffering effects of dissolved protein, and competitive inhibition of proteases by food proteins and added protease inhibitors naturally found in foods (such as the caseins in milk), will enhance bioavailability.

The primary research goals of this stage will be to ensure efficient encapsulation occurs and that transgenic Factor IX activity is recoverable from the formulated product and can be standardized by *in vitro* methods. We will use the standard liposome preparations methods used by Hemker et al. (1980) and Horikoshi et al. (1982) that achieved bioavailabilities in excess of 5% for oral dosage of plasma-derived Factor IX and Factor VIII in dogs and humans. Briefly, a mixture of phospholipids (synthetic or derived from egg lecithin) will be dissolved in ethanol, dried under nitrogen, and reconstituted in an neutral aqueous buffer containing the transgenic Factor IX and protease inhibitors (aprotinin, soybean trypsin inhibitor). At neutral pH, transgenic Factor IX interacts very strongly with alkyl HPLC stationary phases (Van Cott, unpublished observations), and so it is expected that transgenic Factor IX will be incorporated into the liposomes with a high efficiency. Agitation and forced flow through filter membranes will be used to emulsify the mixture. The liposome-encapsulated transgenic Factor IX will be isolated and concentrated using centrifugation. Isolated liposomes will then be lyophilized. After reconstitution, we will quantify the recovery of encapsulated Factor IX activity by aPTT analysis of the liposome fraction isolated by centrifugation.

The formulations will be

1. lyophilized Factor IX liposomes (Hemker et al., 1980), reconstituted in an aqueous syrup, buffered with calcium carbonate, and no added protease inhibitors.

2. lyophilized Factor IX liposomes (Hemker et al., 1980), reconstituted in an aqueous syrup, buffered with calcium carbonate, and added soybean trypsin inhibitor.
3. lyophilized Factor IX liposomes (Hemker et al., 1980), reconstituted in bovine milk (Horikoshi et al., 1982), buffered with calcium carbonate, and no added protease inhibitors.
4. lyophilized Factor IX liposomes (Hemker et al., 1980), reconstituted in bovine milk (Horikoshi et al., 1982), buffered with calcium carbonate, and added soybean trypsin inhibitor.

The aqueous syrup and milk will be used to enhance palatability for younger children and mask the taste of other excipients. USP syrup consists of 85% sucrose (w/v) in water, with a small amount of a preservative (0.1% sodium benzoate). Proteins are known to be stabilized in high concentrations of sucrose. If necessary, modifications of the syrup formulation will include addition of other polyols (mannitol, glycerol).

D.3.2. Postprandial, oral dosage – normal mouse and Factor IX-knockout mouse. The *justification* for these experiments is that the oral dosage formulations in normal and Factor IX-knockout mice will help determine the most efficient formulations for use in normal and hemophilia B dog models. The first *objective* will be to statistically assess the bioavailability of candidate formulations of transgenic Factor IX in normal mice. The second *objective* will be to then assess the most efficient oral dosage formulations in the Factor IX-knockout mouse, which is a more expensive and fragile animal model. The Factor IX-knockout mouse data are needed to evaluate the effectiveness of the transgenic Factor IX in establishing and maintaining hemostasis. Due to cost, we will not assess plasma-derived or BeneFIX® with this oral delivery experimental design.

D.3.2.1. Administration and sampling*.* Lyophilized formulations will be reconstituted as described above and administered postprandially. Single-dose studies will be conducted first, and transgenic Factor IX formulations will be administered by gavage. Long-term studies will consist of treatment for one month with administration every other day. It is desirable to train the animals to drink the formulated transgenic Factor IX from a small Petri dish, as we have done in the past when feeding raw transgenic milk. The goal will be to determine how much transgenic Factor IX must be administered to obtain >5% Factor IX activity levels in the plasma over a 48-72 hour period. A control group of vehicle-only administration will be done in parallel for both normal and Factor IX-knockout mice.

On the day prior to feeding, mice will be tagged and weighed, and a plasma sample will be taken to establish baseline Factor IX clotting activity. Five to seven mice will be used in each treatment group. Post-administration in single-dose pharmacokinetic studies, mice will be anesthetized with intraperitoneal avertin and plasma samples will be taken at 15 min, 1 hour, 4 hours, 24 hours, 48 hours, and 72 hours from retroorbital venous plexus into citrated collection tubes. For long-term studies up to six retroorbital bleeds, plasma samples from anesthetized mice will be taken every three days for an 18-day period. For each subsequent sample, collection will alternate between eyes for retroorbital venous plexus to minimize any effect of partial activation of coagulation *via* tissue factor exposure. The mice will be euthanized at the completion of each study.

D.3.2.2. Factor IX activity and antigen assays. Factor IX antigen and activity levels in the plasma samples and the pharmacokinetics (bioavailability, mean residence time, clearance, etc.) will be determined as described above in Section D.1.4. Samples from normal mice at Colorado State will be shipped to UNC-Chapel Hill or the University of Nebraska-Lincoln for required assays. For normal mouse plasma samples, the Factor IX activity and antigen levels over baseline will be measured, using a pool of normal mouse plasma and the individual mouse plasma samples prior to administration as controls.

D.3.3 Postprandial oral dosage of transgenic Factor IX in normal and hemophilia B dogs. The *justification* for these experiments is that, from the perspective of hemostasis (Brinkhous et al., 1996) and the precedent of preclinical studies in the hemophilia B dog as accepted by the FDA, these data

will be required for our anticipated IND filing. It is also essential that we have a clear understanding of the species difference for oral administration of Factor IX. The first *objective* will be to assess the bioavailability in normal dogs of oral dosage formulations that gave the highest bioavailability in hemophilia B mice. The second *objective* will be to then assess the bioavailability in the hemophilia B dog model of the formulations having the highest bioavailability in normal dogs. Due to cost, plasma-derived Factor IX and BeneFIX® will not be assessed.

D.3.3.1. Postprandial oral administration and sampling. The goal will be to maintain greater than 5% Factor IX activity in plasma for at least 72 hours by oral dosage. In our preliminary work with normal dog giving 500 IU/kg in unformulated raw milk, we achieved similar levels of Factor IX activity in plasma as Horikoshi et al. (1982), who administered 14 U/kg. Because of the expected greater efficiency of absorption of transgenic Factor IX formulated in liposomes, we will bracket our treatment levels between 50-500 IU/kg.

A short-term study will consist of a one-hour, post-prandial administration, followed by sampling over a 72-hour period and then cross-over. An oral dosage cross-over study of vehicle-only and then transgenic Factor IX formulation will be done in both normal and hemophilia B dogs. For normal dogs, treatment groups will be four to five animals for each candidate formulation. For hemophilia B dogs, the treatment group will be three dogs.

A long-term study using the best candidate formulation will consist of post-prandial feedings every two or three days and plasma sampling every three days for 30 days. For both normal dogs and hemophilia B dogs, plasma and serum sampling protocols, coagulation assays, and inhibitor assays will be the same as in Section D.3.2. Samples from normal dogs at Colorado State will be shipped to UNC-Chapel Hill or the University of Nebraska-Lincoln for required assays.

D.4. Program Plan

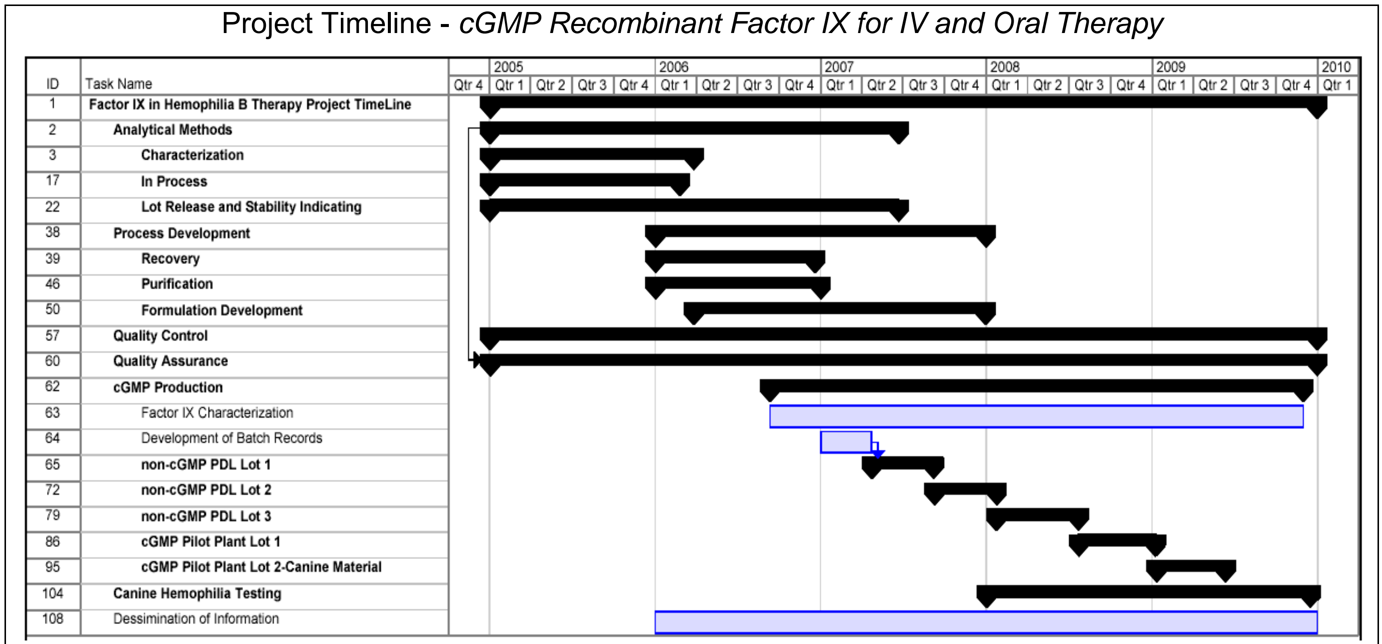
This project will require five years to complete with the final result an evaluation of an oral dosage in a canine hemophilia B model. We have established a well thought-out project time line, which follows. The success of any product destined for clinical trials is a solid foundation of analytical methods that serves four purposes: characterization, in-process testing, stability testing, and methods to release the clinical product. The first 2.5 years will be required to establish all of the assays required to produce cGMP material for the canine studies. In addition, characterization studies (not typically required for final product release, but essential for understanding protein biochemistry) will continue during the entire grant period and are shown as a subtask under the cGMP Task.

Process development will initiate at the beginning of Year 2, which coincides with the establishment and transfer of in-process tests to Quality Control. Two years for process development will be required, with Year 2 dedicated to developing a process to produce bulk material, developing intravenous formulation, and evaluation in hemophilia B mice. We will then be able to initiate the process to produce cGMP material for the canine studies. The first canine studies in Year 4 will be initiated using the first cGMP material. Subsequent cGMP materials will be produced for oral formulation and administration in Year 5. Quality Assurance and Quality Control span the entire project. Having a continuous presence of Quality Assurance during this project is essential to ensure that a quality product is delivered that meets standards for injectables. Including this component in the Program Plan will guarantee a rapid transition into clinical testing.

D.4.1. Major milestones for this project are

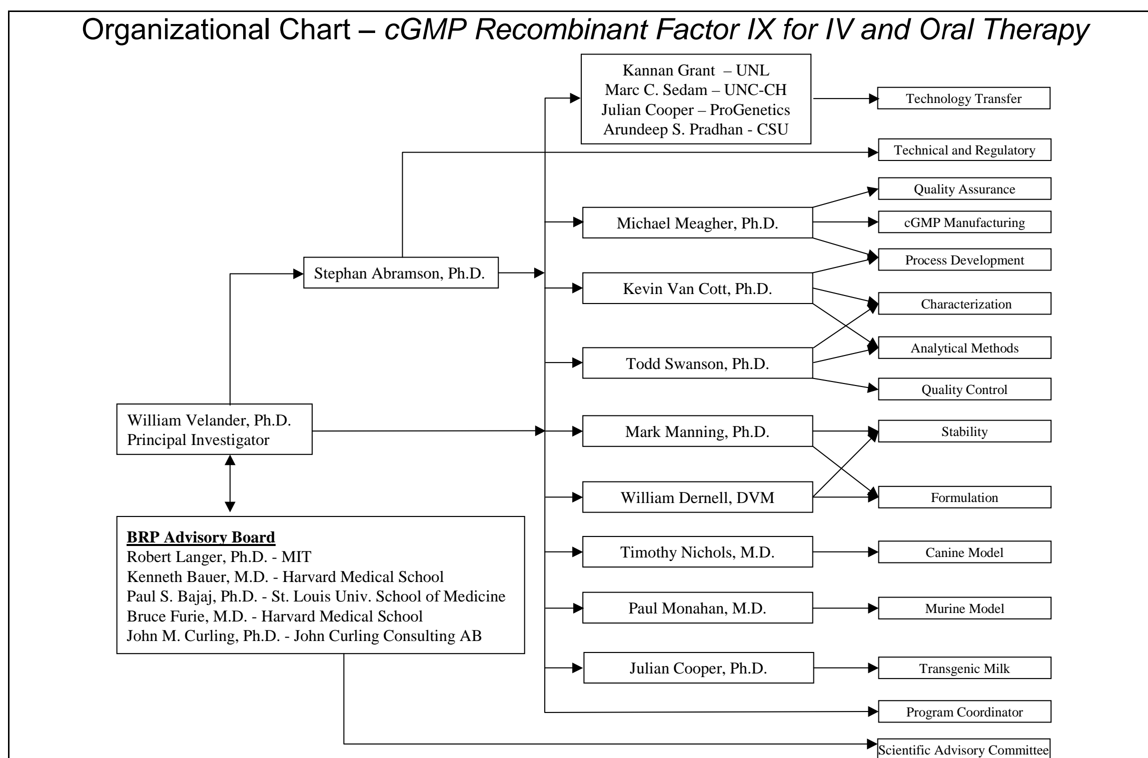
1. Establishing Product Specifications and Analytical Methods to determine these specifications – 2nd Quarter, 2007
2. Establishing the process to produce Factor IX candidate materials – 4th Quarter, 2007
3. First material lot of transgenic Factor IX by cGMP production – 3rd-4th Quarter, 2008

4. Canine hemophilia model – 4th Quarter, 2009



D.5. Organizational Chart and Leadership Statement

Dr. William Velander is the Principal Investigator of this project and has more than 17 years experience and cumulatively more than $6 million of research support in directing transgenic animal engineering research and plasma protein purification research. Dr. Velander will oversee all aspects of this project and coordinate Factor IX characterization, production, and testing in animal models. Dr. Stephan Abramson of LifeSci Partners has two decades of biopharmaceutical product development experience encompassing clinical, technical, and regulatory expertise, including AlphaNine®SD (purified plasma-derived Factor IX). Dr. Abramson will be a Lead Investigator coordinating information needed for future regulatory approval and clinical evaluation. Dr. Julian Cooper of ProGenetics LLC is a Lead Investigator who has extensive experience in the cGMP production of transgenic pigs and sheep for production of biotherapeutics, and will supply FDA- and USDA-compliant Factor IX milk. Dr. Michael Meagher is a Lead Investigator at the University of Nebraska-Lincoln who provides expertise in bioprocess engineering and current Good Manufacturing Practices production of recombinant proteins, and is Director of the Biological Process Development Facility. Dr. Kevin Van Cott is a Lead Investigator presently at Virginia Tech, but will be at the University of Nebraska-Lincoln beginning May 2004. Van Cott will direct characterization and analytical methods development and assist Dr. Todd Swanson in transfer of methods to Quality Control. Dr. Todd Swanson (Key Person) will direct Quality Control. Dr. William Dernell (Key Person) and Dr. Mark Manning (Lead Investigator) of Colorado State will formulate Factor IX products that are produced by the UNL Biological Process Development Facility, and then forward these formulated products to Drs. Paul Monahan and Timothy Nichols at the University of North Carolina at Chapel Hill. Dr. Paul Monahan is a Lead Investigator and will direct hemophilia B mouse model studies. Dr. Timothy Nichols is a Lead Investigator who will direct hemophilia B dog studies. The individuals listed above, along with technology transfer officials from participating institutions, will comprise the *Scientific Steering Group* for this project. The *Scientific Steering Group* will meet monthly (by teleconference) to discuss the progress of the project and any technical issues. An external BRP Advisory Board has been assembled to discuss the progress of the project, suggest alternative strategies if technical problems are encountered, and provide input into future directions that the research team should take.



D.6. Expected Outcomes

It is expected that the success we have had in our preliminary studies will translate to successful cGMP production and successful preclinical evaluation of transgenic Factor IX in the hemophilia B dog model so as to provide rapid transition to Phase I clinical trials. We expect that this success will be derived from the product specifications defined as a result of the work, and that the compliance with these specifications by the transgenic Factor IX product will be supported by in-process, lot release, and characterization assays for the production of Purified Drug Substance and final formulated Product. Thus, it is our strong expectation that a viable intravenous transgenic Factor IX and an oral dosage form of transgenic Factor IX will become available within the projected timeframes for future clinical trials. It is our equal conviction that the same approaches, modified as necessary, will lead to the analogous development and production of recombinant Factor VIII ready to enter advanced development for manufacture of material for Phase I clinical trials.

D.7. Anticipated Problems and Alternative Strategies

Among potential problems is the geographical separation of culturally distinct partners (industry [ProGenetics, LifeSci Partners], academe [University of Nebraska-Lincoln and University of North Carolina at Chapel Hill, Colorado State University], and government (NIH NHLBI). This critical issue has been solved by making the University of Nebraska-Lincoln the lead institution on this proposal as a result of its extensive experience producing cGMP biologics with collaborators of similar geographical separation. The Principal Investigator, Dr. William Velander (UNL), is a co-architect of the FDA's *Points to Consider in the Manufacture and Testing of Therapeutic Products for Human Use Derived from Transgenic Animals,* and has biopharmaceutical experience of plasma-derived Protein C and Factor IX at pilot plant scale of 5,000 Liter batches with the American Red Cross Holland Laboratories, and is, therefore, ideal as the project leader. The Lead Investigators at UNL are demonstrably ready and able to coordinate the efforts required for success in this joint effort to produce the transgenic Factor IX products. Dr. Velander's efforts will be facilitated by the fact that most of the investigators have

experience working together and have a long-term interest in accomplishing the overall goal of this application. As evidenced in our preliminary studies, the partners have a track record of working and problem-solving together over for a long period of time, and the same approach will underpin this effort. Of equal importance will also be the integration of hemophilia treatment staff of the National Heart Lung and Blood Institute.

Interdependence of the partners is a strength and, at the same time, a potential weakness. Strength lies in the fact that the whole is greater than the sum of the parts; no one of the groups could accomplish the overall goal in the same time without the other partners. Potential weakness lies in the fact that loss of one of the key partners, although a remote possibility, could interrupt the overall effort for a brief period. However, given the quality of Lead Investigators and Key Persons working in the program, coupled with high-quality record-keeping, data management, and availability, the loss of any one key individual would not translate into loss of knowledge and, in most cases, little if any time would be lost. In addition, appropriately qualified individuals will be recruited either from within each partner organization or from external sources in a timely manner. All information generated by all sites will be shared among the project team electronically. Each site will be required to provide a monthly summary of results to the Program Coordinator who will assemble these documents into monthly project summary reports. These reports will be very useful for technology transfer into cGMP and to assist in the writing later on of the Investigational New Drug (IND) application. The monthly reports will serve as a basis for mandatory reports to the NIH program director. All of the reports will reside on the CyberLab system at UNL and will be accessible to all participating institutions through the Web. The NIH program director will also have access to this information. The NIH program director is welcome to participate in the weekly phone conferences as well.

Although we do not anticipate issues with the current transgenic pig source material, it is possible that the current lineages may not be suitable for producing clinical grade material, and new lineages may need to be made. This is not viewed as a significant hurdle based on our 17 years of experience in producing transgenic pigs. In the case of purification, capture is viewed as the most time-consuming step in process development and presents the greatest challenges. Although it is difficult to present a solution, the combined experience of this cooperative team will be applied to solve this problem. This group has more combined experience with Factor IX and transgenically expressed, post-translationally complex proteins than any group in the world. Detailed immunologic studies concerning tolerization are beyond the scope of funding for this project, but our collaborators at NIH NIAID will be pursuing separate funding.

The Biological Process Development Facility's experience with companies that request rapid transition of their drug candidate into Phase I clinical trials is that yield and sometimes scalability of the process are sacrificed at the expense of "getting enough" material for Phase I studies. Successful Phase I studies translates into an infusion of capital by investors and then the process is "fixed." In the case of transgenic Factor IX, this model does not apply, because there is a very high probability that transgenic Factor IX will succeed. Eventual Phase I clinical studies will be performed with hemophilia B patients, thus, a consistent supply of transgenic Factor IX must be guaranteed so treatment can continue past Phase I. The BPDF will be able to build on the existing knowledge of BeneFIX® and, more importantly, the plasma-derived AlphaNine®SD, because Dr. Stephan Abramson was directly involved in the organization of clinical trials and commercialization of AlphaNine®SD. Given all of these factors and the fact that commercial-scale is actually pilot-scale, process development at the BPDF will be focused on developing a process suitable for commercial production.

At the end of the proposed investigations, it is expected that transgenic Factor IX will be formulated into a viable product for intravenous therapy and for oral dosage. It is, however, recognized that these formulations, while starting from the same source material, will be pharmaceutically different, and there is the possibility that additional expertise and new technology for oral delivery may be needed to increase efficiency of bioavailability for adult oral administration because of the large number of units

that may be needed. In that case, it is possible that only oral dosage for infants may be feasible in the context of the proposed budget and research plan.

Finally, we have assembled an external and independent BRP Advisory Board consisting of individuals that are experts in biomedical engineering of drug delivery, Factor IX biochemistry, hemophilia treatment, and bioprocess engineering of blood plasma proteins and recombinant proteins. These individuals have no financial interests in the project and will provide an objective evaluation of the progress of the project.

- Robert Langer, Ph.D. (Massachusetts Institute of Technology) is a biomedical engineer and an expert in drug delivery.
- Kenneth Bauer, M.D. (Harvard Medical School) is a clinician and expert in coagulopathic diseases including those related to Factor IX.
- Paul S. Bajaj, Ph.D. (St. Louis University School of Medicine) is an expert in Factor IX biochemistry.
- Bruce Furie, M.D. (Harvard Medical School) is an expert in recombinant manipulations of Factor IX.
- John M. Curling, Ph.D. (John Curling Consulting AB) is an expert in process purification and production of therapeutic proteins derived from plasma and recombinant sources.

D.8. Technology Transfer Integration Plan

Technology transfer can be a major hurdle for inexperienced groups. Such problems are highly unlikely for the group proposed here, because the principal partners (ProGenetics, LifeSci Partners, University of Nebraska-Lincoln, University of North Carolina at Chapel Hill, and Colorado State) have direct, successful experience with biologics production. All are familiar and experienced with operating within the framework of Good Laboratory Practices and current Good Manufacturing Practices. In the unlikely event that such problems were to be encountered, additional managerial emphasis would be given to overcoming the difficulties and to making sure that they did not continue to be a problem.

Intellectual property issues and the issue of who will get the credit for successful attainment of the overall goal have been discussed openly and solved. All partners are prospectively in agreement that their own interests will be subordinated to that of attaining the overall goal. Clear agreements are in place as to how intellectual property and credit for discovery will be assigned. Technology transfer officials from ProGenetics LLC (Julian Cooper), University of Nebraska-Lincoln (Kannan Grant), University of North Carolina at Chapel Hill (Marc Sedam), and Colorado State University (TBN) will be included as members of the *Scientific Steering Group*.

D.9. Data Sharing Plan

Data generated during this project will be published in peer-reviewed journals and presented at national and international meetings. Lead and corresponding authors of articles and presenters of technical talks will acknowledge all of the project team members who contributed to the work. Assignment of lead and corresponding authorship will be agreed upon by the Scientific Steering Group. Data generated in this project that are not proprietary will be made available to the public at the University of Nebraska-Lincoln Biological Process Development Facility web site (http://bpdf.unl.edu). Data that will be posted on the web site will include slides from technical presentations and seminars, reprints of journal articles and conference proceedings, and links to the National Hemophilia Foundation and World Federation of Hemophilia web sites.

D.10. Dissemination Plan

The audiences most suitable for viewing the research results of this project are the hemophiliac community and clinicians treating hemophilia. Our activities to reach this audience will include travel to national meetings and international meetings for the World Federation of Hemophilia, the American Society of Hematology, and the National Hemophilia Foundation. Dr. Velander will be giving an invited plenary talk at the WFH meeting in Thailand in 2004 and has routinely given talks to the NHF Gene Therapy workshops and the Hemophilia World Congress meetings. Travel money is requested to support these activities throughout the duration of the project. In addition, slides and brochures from these presentations will be made available on our web site, with links to the web sites of the National Hemophilia Foundation and the World Federation of Hemophilia. Part of the Program Coordinator's and IT support staff's duties will be to ensure that our web site at the University of Nebraska-Lincoln is maintained and up-to-date.

D.11. Future Directions

Prior to human clinical trials, there will be additional preclinical studies such as hypercoagulopathic potential evaluated in the Wessler rabbit model and pigs. In addition, long-term pharmacokinetic and immunologic studies, especially for oral dosage forms with gel capsules, will be needed. These studies are beyond the scope of funding for the current five-year BRP project. However, at the completion of the presently proposed studies, we will make an application for studying long-term pharmacokinetic and immunologic effects of transgenic Factor IX. In addition, validation of viral pathogen reduction will be required prior to clinical trials. There are also additional regulatory requirements involving process validation prior to clinical trials that are beyond the scope of this funding.

We anticipate that the successful production of clinical-grade transgenic Factor IX and proof in the hemophilia B dog animal model of a quality that includes long half-life and an abundance that will enable prophylaxis to be explored worldwide will serve as a blueprint for analogous work with Factor VIII and von Willebrand Factor (vWF). Our confidence in this direction derives from our successful work with the expression of Factor VIII and vWF in the milk of transgenic pigs and mice, respectively (Paleyanda et al., 1997; Velander et al., unpublished results). This work includes the bioengineering necessary to optimize the post-translational modifications using the balance of genetic control over expression levels of the target protein as well as the insertion of recombinant post-translational processing enzymes such as furin (Drews et al., 1995). While we are confident that even with the low bioavailability, oral administration will be practical for infants of hemophilia A and B, as discussed previously. Based upon expression of new molecular designs of Factor VIII that overcome restricted secretion, we expect to have a supply of Factor VIII-containing milk produced in pigs within two years. We also expect to have vWF-containing pig milk within two years.

E. Human Subjects

Not applicable

F. Vertebrate Animals

F.1. Provide a detailed description of the proposed use of the animals in the work outlined in the Research Design and Methods section. Identify the species, strains, ages, sex, and numbers of animals to be used in the proposed work.

ProGenetics: Transgenic pigs. IACUC approval number and date: pending; Animal welfare assurance number: 52-R-0108.

The project would require the use of five adult transgenic female pigs (*Sus. scrofa*). Animals will be of normal breeding age (eight months) or older. The pigs will be bred through artificial insemination as per

standard agriculture technique. Animals will be maintained through gestation and allowed to farrow per industry standard.

Colorado State University. IACUC approval number and date: pending; Animal welfare assurance number: A3572-01.
Animals (dogs, mice) will be purchased from class A vendors; the specific vendor will be chosen based on availability. Specific strains will be as follows: a) dogs will be Walker hounds and b) mice will be C3HeJ. These strains are chosen for their hardiness and our familiarity with their response to procedures performed. All animals will be examined upon arrival to determine health status. A complete blood count, biochemical profile, and urinalysis will be performed on each dog to obtain baseline values. Upon arrival at Colorado State University (CSU), all animals will be allowed to acclimate for 7 days (minimum) prior to additional procedures being performed. All animals will be housed at the laboratory animal facilities at the CSU James L. Voss Veterinary Teaching Hospital. Dogs will be adult animals between 1-2 years of age, weighing approximately 10 kg. We expect to use 3-4 animals in each treatment group for testing each oral formulation - about 12 dogs total. For mice, we will use about 60 total animals, 4-6 weeks old.

UNC-Chapel Hill: Hemophilia B mice. IACUC approval number and date: pending (application number is 04-002.0A); Animal welfare assurance number: A-3410-01.
The following strains of mice will be maintained at the Gene Therapy Center of the University of North Carolina at Chapel Hill for these studies: normal C57BL/6, normal BalbC, and hemophilia B (Factor IX knockout mice in C57BL/6, BalbC, and mixed C57BL/129 strain backgrounds). We will use mice between 2 and 5 months old for experiments and will maintain breeding pairs between 2-12 months of age. We will use 12 breeding pairs plus four to five treatment groups of seven to eight mice in each budgeted year.

UNC-Chapel Hill: Hemophilia B dogs. IACUC approval number and date: 02-135.0-B, 1-7-04; Animal welfare assurance number: A-3410-01.
The following phenotypes of the dogs will be maintained at the Francis Owen Blood Research Laboratory at the University of North Carolina at Chapel Hill for these studies: normal and hemophilia B (Factor IX deficient). We will use male and female dogs predominantly between 2 and 12 months old for experiments, and older dogs as breeders and blood product donors. Each hemophilic dog has an average of ~six bleeds per year that require these blood products (Russell et al., 2003). We anticipate maintaining an average census of 10 to 20 dogs and including 10 to 20 puppies. The goal of this grant application is to test Factor IX made in transgenic pigs for its ability to correct the hemophilic coagulopathy. The hypothesis we are testing is that replacement of the missing proteins with these new transgenic Factor IX formulations will correct the bleeder phenotype. In addition to the use of these animals in the described protocols, blood will be used in the *in vitro* studies and tissue in *ex vivo* studies.

F.2. Justify the use of animals, the choice of species, and the numbers to be used. If animals are in short supply, costly, or to be used in large numbers, provide an additional rationale for their selection and numbers.

ProGenetics: Transgenic pigs. Previous research of transgenic swine has indicated their ability to produce and secrete functional vitamin K depend proteins into milk. Currently, ProGenetics LLC maintains a herd of transgenic swine at its class R research facility (USDA # 19485) that produces transgenic Factor IX. Results from previous research have indicated the production capability of these transgenic swine under the current milking protocol to be 100 liters per year. While only one animal is need to fulfill the required amount of milk need for this project, a pool from at least five animals will be used to ensure reproducibility.

Colorado State University. Animals (dogs, mice) will be purchased from class A vendors; the specific vendor will be chosen based on availability. Specific strains will be as follows: a) dogs will be Walker hounds and b) mice will be C3HeJ. These strains are chosen for their hardiness and our familiarity with their response to procedures performed. The number of animals that we have specified, 12 dogs and about 60 mice, are needed to obtain a statistical significance in the evaluation of each oral formulation.

UNC-Chapel Hill: Hemophilia B mice. We will examine the ability of transgenic pig-derived recombinant human Factor IX, when compared with commercially available human plasma-derived or CHO cell-derived Factor IX, to function in the blood coagulation cascade. To adequately compare efficacy of the new human Factor IX preparation with the currently available products, testing *in vivo* the coordinated interactions of the clotting system (coagulation Factors I-XIII and von Willebrand factor interacting with platelets and the vascular endothelial system) is required. In addition, an intact immune system is required for comparative testing of the incidence of anti-Factor IX humoral immune responses ("inhibitor antibody" development) with the transgenic-derived Factor IX as compared to available products. To do this, we may need to characterize and vary the immune background of the recipient species (requiring inbred strains of mice) and the genotype of the disease model (requiring transgenic hemophilic mice), while keeping intact the immune and coagulation systems. A non-animal alternative is not available. We will use 12 breeding pairs plus four to five treatment groups of seven to eight mice in each budgeted year. This number of animals is required to obtain the statistical significance of pharmacokinetic properties in the mice.

The hemophilia B mouse model was created at the University of North Carolina by Dr. Darrel Stafford, using a strategy of homologous recombination that knocked out the promoter through the fourth exon of the mouse Factor IX gene. The mouse has no Factor IX activity and has no circulating Factor IX protein. As a result, the mouse has a bleeding tendency that resembles human Factor IX deficiency. Specifically, the mouse has abnormal (prolonged) *in vitro* blood clotting results, joint and muscle hemorrhages with trauma, and both difficulty forming a stable initial clotting and late re-bleeding after injury. The hypothesis we are testing is that replacement of the missing mouse Factor IX with the new transgenic Factor IX formulations will correct the bleeder phenotype. In addition, we will test the hypothesis that transgenic Factor IX, when compared to commercially available Factor IX products, will not be more immunogenic; i.e., transgenic Factor IX will be no more likely to stimulate the development of anti-Factor IX inhibitor antibodies in the hemophilic mouse.

UNC-Chapel Hill: Hemophilia B dogs. We have chosen hemophilic dogs for this study for several reasons. First, the bleeder phenotype of these dogs recapitulates the human disease of the same type. Second, other related products that have been successful at correcting the hemophilic coagulopathy in these dogs have also been found to work in human hemophiliacs. The availability of an established animal model of hemophilia with a track record of predicting success in treating human hemophiliacs is an important facet of this grant application. Murine models of hemophilia B are also available and will be used for screening various Factor IX formulations. However, an average mouse weighs 25 grams and has a circulating blood volume of ~1.5 cc. This small size limits the volume and frequency of blood drawing. Frequent blood sampling is essential in these studies because we are trying to determine the half-life of these new Factor IX products. The availability of these dogs with these well-characterized bleeding disorders represents a unique opportunity to perform these preclinical studies to treat deficiencies in blood coagulation proteins. The number of dogs chosen is the number required to ensure the availability of experimental and control dogs. Our strategy for accomplishing this goal is to maintain three dogs for every two experimental and control dogs to ensure that an orderly sequence of studies can be conducted without costly delays.

F.3. Provide information on the veterinary care of the animals involved.

ProGenetics: Transgenic pigs. Animals will be inspected at least twice annually by a certified veterinarian that specializes in large animal medicine. Currently, ProGenetics LLC has a contractual

arrangement with Bold Springs Veterinary Services (Blacksburg, Virginia) for both emergency and non-emergency care. The attending veterinarian has established and maintained programs of disease control and prevention, pest and parasite control, procedural care, nutrition, and euthanasia on the premises of ProGenetics LLC.

Colorado State University. All animals will be handled and cared for by trained personnel, either from the Dr. John Venners Pharmacology laboratory or Laboratory Animal Resources at CSU according to the GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS (National Institutes of Health publication no 85-23). Laboratory animal facilities at CSU are accredited by the American Association for the Accreditation of Laboratory Animal Care. Dogs will be housed in elevated runs and fed a standard dry ration. Dogs will be allowed 7 days to acclimate to their surroundings prior to experiments. Mice will be housed in an AALAC-approved facility in cages of five animals each, and kept in a temperature-controlled environment with 12 hour cycles of light and darkness. Animal care at the facility is designed and supervised by the veterinary staff of the Division of Laboratory Animal Medicine, including Dr. Don Maul, who is a board-certified laboratory animal veterinarian. The majority of the veterinary care and supervision at the laboratory will be performed by Dr. Dernell, the laboratory co-director and a licensed veterinarian.

UNC-Chapel Hill: Hemophilia B mice. Mouse breeding and experiments will be conducted under the direct supervision of Dr. Monahan and under the auspices of the Division of Laboratory Animal Medicine at the University of North Carolina. Mice are maintained in a virus- and pathogen-free facility of the University of North Carolina Department of Laboratory Animal Medicine in the building of the Lead Investigator (Monahan). Animal care at the facility is designed and supervised by the veterinary staff of the Division of Laboratory Animal Medicine, including Dr. Dwight Bellinger, who is a board-certified laboratory animal veterinarian. Dr. Bellinger has more than 20 years experience in the use of animals for study of human coagulation proteins and advises experimental design in mice as well as in the colonies of hemophilic canines maintained at the UNC-CH Francis Owen Blood Research Laboratory.

UNC-Chapel Hill: Hemophilia B dogs. The dogs will be raised under the auspices of the Division of Laboratory Animal Medicine at the University of North Carolina. The dogs are born and reared at the Francis Owen Blood Research Laboratory, a facility of The University of North Carolina Department of Pathology and Laboratory Medicine. This laboratory has been dedicated to the production and care of dogs and pigs with inherited bleeding disorders for more than 40 years. The laboratory is accredited by the American Association for the Accreditation of Laboratory Animal Care and operates according to the GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS (National Institutes of Health publication no 85-23). Animal care at the facility is designed and supervised by the veterinary staff of the Division of Laboratory Animal Medicine, including Dr. Dwight Bellinger, who is a board-certified laboratory animal veterinarian. The majority of the veterinary care and supervision at the laboratory is done by Dr. Bellinger who has done this work for more than 20 years.

F.4. Describe the procedures for ensuring that discomfort, distress, pain, and injury will be limited to that which is unavoidable in the conduct of scientifically sound research. Describe the use of analgesic, anesthetic, and tranquilizing drugs and/or comfortable restraining devices, where appropriate, to minimize discomfort, distress, pain, and injury.

ProGenetics: Transgenic pigs. Transgenic pigs used in this study will not be subjected to discomfort, distress, pain, or injury. Animals will be allowed free access to food and water during the procedure. During the past 17 years, we have shown that piglets of lactating females that are milked up to five times/day have normal growth rates when provided with industry-standard milk replacement.

Colorado State University. Sedation or anesthesia will be used to minimize discomfort and distress when necessary. Animals are handled by experienced personnel and anesthesia is supervised by professional staff.
Dogs: Morphine (1-2 mg/kg subcutaneously) and acepromazine (0.01-.2mg/kg subcutaneously) is used to sedate the dogs for minor procedures such as intravenous injections and blood withdrawal.
Mice: Intravenous (tail vein) injections will be performed under isoflourane gas anesthesia by nose cone (mask) induction and maintenance.

UNC-Chapel Hill: Hemophilia B mice. Anesthesia and sedation will be used to minimize discomfort and distress. Animals are handled by experienced personnel whose proficiency with procedures is evaluated by the Compliance Coordinators of the Office of Institutional Animal Care and Use of UNC-CH. For minor procedures (e.g., phlebotomy) anesthesia is accomplished with inhaled isoflurane to effect. For surgical procedures anesthesia is accomplished by giving avertin 0.017-.018 ml/gram or inhaled isoflurane to effect. Anesthesia is evaluated by respiratory rate and persistence or absence of withdrawal reflexes (toe pinch withdrawal).

UNC-Chapel Hill: Hemophilia B dogs. Anesthesia and sedation will be used to minimize discomfort and distress. Animals are handled by experienced personnel and anesthesia is supervised by professional staff. Ketamine (10/mg/IM) or Medetomidine -HCl 750 µg/m BSA IV is used to sedate the dogs for minor procedures such as blood withdrawal and determining bleeding times. For surgical procedures anesthesia is accomplished by giving Medetomidine -HCl 750 µg/m BSA IV followed by isoflurane to effect (maintenance at around 2%). Nitrous oxide (50%) is occasionally used during induction. Anesthesia is evaluated by heart rate, blood pressure, and persistence or absence of palpebral, corneal, and withdrawal reflexes.

F.5. Describe any method of euthanasia to be used and the reasons for its selection. State whether this method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. If not, present a justification for not following the recommendations.

ProGenetics: Transgenic pigs. Euthanasia is performed with an overdose of TKX to render the animal unconscious, followed by a lethal electric shock. This method is chosen because it is quick and non-stressful to the animals and because it is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Colorado State University. Euthanasia (dogs) is performed with an overdose of pentobarbital (88 mg/kg intravenously). Euthanasia (mice) will be performed by cervical dislocation under isoflourane anesthesia. These methods are chosen because they are quick and non-stressful to the animals and because they are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

UNC-Chapel Hill: Hemophilia B mice. Euthanasia is performed with an overdose of avertin (0.035 ml/gm body weight) or ketamine (0.2 mg/gram body weight), followed by cervical dislocation. This method is chosen because it is quick and non-stressful to the animals and because it is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

UNC-Chapel Hill: Hemophilia B dogs. Euthanasia is performed with an overdose of pentobarbital (6 grains/10 lbs). This method was chosen because it is quick and non-stressful to the animals and because it is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

G. Literature Cited

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Colorado State University
Knowledge to Go Places

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Dr, William Velander
Department of Chemical Engineering
207 Othmer Hall
PO Box 880643
University of Nebraska-Lincoln
Lincoln, NE 68588-0643

January 20, 2004

Dear Dr. Velander

This letter is in enthusiastic support of your proposed work entitled *cGMP Recombinant FIX for IV & Oral Hemophilia B Therapy* which will be submitted to the NIH Division of Blood Diseases and Resources (NHLBI/DBDR). We are excited to be a part of this study investigating a novel method of synthesis of factor IX which would have a tremendous impact in the worldwide treatment of hemophilia. We understand that our portion of the proposed work will involve both oral and intravenous formulation work, screening of these formulations for bioavailability using pharmacokinetic evaluation in normal animal systems (mice and dogs). Our laboratory also has expertise in using cell culture systems and a perfused intestinal model that we can use to screen formulations, if necessary. Our laboratory has a long history of drug discovery work and extensive experience and expertise in cell culture and animal models for in vitro and in vivo evaluation of pharmaceuticals. We have expanded our experience and capabilities in tumor cell culture and in vivo animal model systems and are able to offer the facilities necessary for continuation of this type of research. We feel our experience in this area will add valuable expertise to the in vitro and in vivo portion of your proposed work. The questions that you seek to answer are important in the overall effort to treat this devastating disease.

Sincerely,

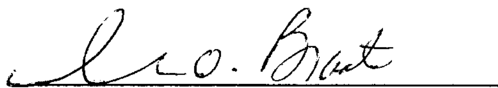
William S. Dernell DVM, MS
Associate Professor, Surgical Oncology
Co-Director, Dr. John Venners Pharmacology Laboratory
Animal Cancer Center

INTENT TO FORM A CONSORTIUM AGREEMENT

TITLE OF APPLICATION:	cGMP Recombinant Human Factor IX in Hemophilia B Therapy
SUBMITTED TO:	The National Institutes of Health
APPLICANT ORGANIZATION:	University of Nebraska-Lincoln
PERFORMANCE SITES:	University of Nebraska-Lincoln Lincoln, NE Investigator: Dr. William Velander LifeSci Partners Altadena, CA

The appropriate programmatic and administrative personnel at each organization involved in this grant application are aware of the PHS/NIH consortium grant policy and are prepared to establish the necessary inter-institutional agreements consistent with that policy.

For the Board of Regents, University Nebraska, University of Nebraska-Lincoln:



Norman O. Braaten
Director, Pre-Award Development

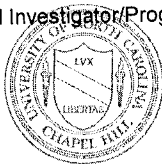
Date: 1/15/04

For LifeSci Partners

Stephan B. Abramson, Ph.D.
Principal

Date: 10 JAN 04

UNL LifeSci Intent 09 Jan04



THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL
Office of the Vice Chancellor for Research and Economic Development

Office of Sponsored Research
Campus Box 1350, 440 W Franklin St
Chapel Hill, NC 27599-1350
(919) 966-3411 FAX: (919) 962-5011

January 6, 2004

Norman Braaten
University of Nebraska at Lincoln
Office of Sponsored Research
312 N 14 Street
Alexander Bldg. West
Lincoln, NE 68588-0430
(402) 472-9392

Subject: Letter of Intent

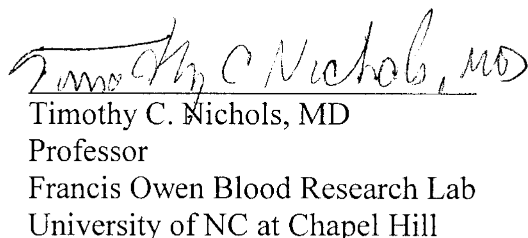
Dear Mr. Braaten;

The University of North Carolina at Chapel Hill (NC-CH), Francis Owen Blood Research Lab are prepared to establish a written agreement with University of Nebraska at Lincoln to work on the project entitled "cGMP Recombinant Human Factor IX in Hemophilia B Therapy." UNC-CH is committed to service within the budget estimate of $180,000 year 3, $180,000 year 4 and $200,000 in year 5, for a total of $560,000 over a three year period.

Animal approval will be obtained from the UNC-CH office.

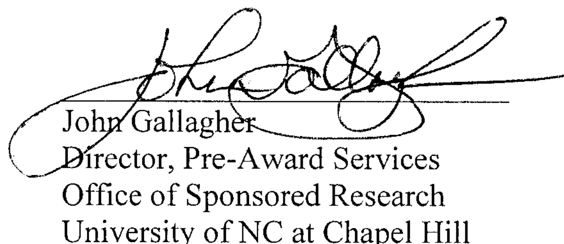
If you have any questions regarding this matter, please John Gallagher at (919) 843-0874.

Principal Investigator



Timothy C. Nichols, MD
Professor
Francis Owen Blood Research Lab
University of NC at Chapel Hill

Authorized Official



John Gallagher
Director, Pre-Award Services
Office of Sponsored Research
University of NC at Chapel Hill

THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL
Office of the Vice Chancellor for Research and Economic Development

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January 14, 2004

Norman Braaten
University of Nebraska at Lincoln
Office of Sponsored Research
312 N 14 Street
Alexander Bldg. West
Lincoln, NE 68588-0430
(402) 472-9392

Subject: Letter of Intent

Dear Mr. Braaten;

The University of North Carolina at Chapel Hill (UNC-CH), Gene Therapy Center, is prepared to establish a written agreement with University of Nebraska at Lincoln to work on the project entitled "cGMP Recombinant Human Factor IX in Hemophilia B Therapy." UNC-CH is committed to service within the budget estimate of $51,744(direct costs) and $23,802 (indirect costs)= $75,546 year 2, $54,971 (direct costs) and $25,287 (indirect costs) = $80,258 year 3 and $56,566 (direct costs) and $26,020 (indirect costs) = $82,586 year 4, for a total of $163,281(direct costs) and $75,109(indirect costs) = $238,390 direct and indirect costs for years 2, 3, and 4.

Animal approval will be obtained from the UNC-CH office.

If you have any questions regarding this matter, please John Gallagher at (919) 843-0874.

Principal Investigator

Paul Monahan, MD
Assistant Professor
Gene Therapy Center
University of NC at Chapel Hill

Authorized Official

John Gallagher
Director, Pre-Award Services
Office of Sponsored Research
University of NC at Chapel Hill

University of Nebraska Lincoln

VICE CHANCELLOR FOR RESEARCH AND DEAN OF GRADUATE STUDIES

January 16, 2004

Dr. Richard E. Swaja
National Institute of Biomedical Imaging and Bioengineering
National Institutes of Health/DHHS
6707 Democracy Blvd., Suite 200
Bethesda, MD 20892-5469

Dear Dr. Swaja:

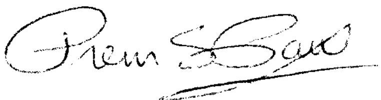
The University of Nebraska-Lincoln is pleased to submit the proposal "cGMP Recombinant FIX for IV and Oral Hemophilia B Therapy" to the Bioengineering Research Partnerships program. We are eager to integrate our expertise in bioprocess and transgenic animal engineering with the strengths of our partners to develop an abundant, safe, and effective therapy for people who suffer from the debilitating effects of hemophilia.

The Bioengineering Research Partnerships program aligns well with UNL's strategic plan to develop priority research areas and to build integrated multidisciplinary collaborations with other academic institutions and industry. A key strength of this application is UNL's unique position as the only academic institution that combines a Biological Process Development Facility with the ability to make complex genetically engineered therapeutic proteins. Another strength is the leadership of Dr. Bill Velander, who was instrumental in the formulation of federal regulatory guidelines for human therapeutics derived from transgenic animals and is a co-inventor of several patents concerning gene transfer and the production of recombinant proteins.

UNL is strongly committed to bioengineering research and, despite the severe state budget deficits of the past two years, we have allocated substantial funds to support development of our research programs in this area. The bioengineering program has been designated as one of 14 UNL Programs of Excellence and has been allocated $493,000 over three years under that program. In addition, we provided $300,000 for equipment and laboratory facilities as part of Dr. Velander's start-up package in 2003.

Thank you for your consideration of our proposal. We look forward to working with our partners and NIH to make this greatly needed therapy a reality.

Sincerely,



Prem S. Paul, D.V.M., Ph.D.
Vice Chancellor for Research
& Dean of Graduate Studies

302 Canfield Administration Building / P.O. Box 880433 / Lincoln, NE 68588-0433 / (402) 472-3123 / FAX (402) 472-3834

William H. Velander, Ph.D.
Professor and Chairperson
Department of Chemical Engineering
University of Nebraska-Lincoln
207 Othmer Hall
Lincoln, NE 68588-0642

9th January 2004

Dear Bill

Thank you very much for asking ProGenetics to be involved with this exciting project to develop a new, safe and inexpensive recombinant form of human factor IX.

ProGenetics will be happy to supply your group with human FIX transgenic pig milk for the duration of the project and will also provide information on the animals' history and health status. We should be able to supply up to 100 liters of whole, fresh frozen pig milk per year, having an average Factor IX specific activity of greater than 100 units per ml. In compliance with the USFDA, which regulates the transport and disposal of transgenic animals and milk, we have established an NIAD file and we will supply the necessary shipping-tracking record forms.

We are very eager to participate in this important work and look forward to working with your group. I will be happy to act as an investigator and am pleased with the organizational chart that you have constructed. Your scientific advisory board is also impressive and should give excellent and objective direction to the project. Please let me know if there is anything else I, or ProGenetics, can contribute to the project.

Best wishes,

Julian D. Cooper PhD
Chief Executive Officer
ProGenetics LLC

1872 Pratt Drive, Suite 1400 • Blacksburg, Virginia 24060 • Tel: 540 961 1680 • Fax: 540 961 1681 • e-mail: julian.cooper@progtx.com

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

[X] NEW application. *(This application is being submitted to the PHS for the first time.)*

[ ] SBIR Phase I [ ] SBIR Phase II: SBIR Phase I Grant No. ______ [ ] SBIR Fast Track

[ ] STTR Phase I [ ] STTR Phase II: STTR Phase I Grant No. ______ [ ] STTR Fast Track

[ ] REVISION of application number: ______
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

[ ] COMPETING CONTINUATION of grant number: ______
(This application is to extend a funded grant beyond its current project period.)

[ ] SUPPLEMENT to grant number: ______
(This application is for additional funds to supplement a currently funded grant.)

INVENTIONS AND PATENTS
(Competing continuation appl. and Phase II only)

[ ] No

[ ] Yes. If "Yes," [ ] Previously reported [ ] Not previously reported

[ ] CHANGE of principal investigator/program director.
Name of former principal investigator/program director: ______

[ ] FOREIGN application or significant foreign component.

1. PROGRAM INCOME ***(See instructions.)***
All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)
	$0.00	

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***
The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/ certifications are provided in Section III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects; •Research Using Human Embryonic Stem Cells• •Research on Transplantation of Human Fetal Tissue •Women and Minority Inclusion Policy •Inclusion of Children Policy• Vertebrate Animals• •Debarment and Suspension; •Drug- Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Non-Delinquency on Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA and Human Gene Transfer Research; •Financial Conflict of Interest (except Phase I SBIR/STTR) •STTR ONLY: Certification of Research Institution Participation.

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions.

[X] DHHS Agreement dated: September 4, 2003 [ ] No Facilities And Administrative Costs Requested.

[ ] DHHS Agreement being negotiated with ______ Regional Office.

[ ] No DHHS Agreement, but rate established with ______ Date ______

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)*

a. Initial budget period:	Amount of base $	995,906	x Rate applied	45.50%	= F&A costs	$	453,137
b. 02 year	Amount of base $	1,104,852	x Rate applied	45.50%	= F&A costs	$	502,708
c. 03 year	Amount of base $	1,014,939	x Rate applied	45.50%	= F&A costs	$	461,797
d. 04 year	Amount of base $	1,155,948	x Rate applied	45.50%	= F&A costs	$	525,956
e. 05 year	Amount of base $	1,044,375	x Rate applied	45.50%	= F&A costs	$	475,191
					TOTAL F&A Costs $		2,418,789

*Check appropriate box(es):

[ ] Salary and wages base [X] Modified total direct cost base [ ] Other base *(Explain)*

[ ] Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.)*:

4. SMOKE-FREE WORKPLACE [X] Yes [ ] No *(The response to this question has no impact on the review or funding of this application.)*

Explanation of subaward F&A costs:

Subaward F&A	**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Year 5**	**Totals**
LifeSci Partners (44%)	37,906	39,043	40,214	41,421	42,664	201,248
CSU (45%)	51,893	80,192	0	0	0	132,085
UNC (combined) (46%)	23,693	0	83,063	26,148	75,437	208,341
Progenetics (45%)	56,637	0	29,599	30,487	31,402	148,125
Total subs F&A	170,129	119,235	152,876	98,056	149,503	689,799

Appendix

Lindsay M, Gil G-C, Cadiz A, Velander WH, Zhang C, Van Cott KE. Purification of recombinant DNA-derived factor IX produced in transgenic pig milk and fractionation of active and inactive subpopulations. *J. Chromatogr*. 2004; 1026:149-157.

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Purification of recombinant DNA-derived factor IX produced in transgenic pig milk and fractionation of active and inactive subpopulations

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Abstract

Transgenic animal bioreactors can be engineered to make gram per liter quantities of complex recombinant glycoproteins in milk. However, little is known about the limitations in post-translational processing that occurs for very complex proteins and how this impacts the task of purification. We report on the purification of recombinant factor IX (rFIX) from the milk of transgenic pigs having an expression level of 2–3 g rFIX/(l^{-1} h^{-1}), an expression level that is about 20-fold higher than previously reported. This purification process efficiently recovers highly active rFIX and shows that even complex mixtures like pig milk, which contains 60 g/l total endogenous milk protein and multiple subpopulations of rFIX, can be processed using conventional, non-immunoaffinity chromatographic methods. Without prior removal of caseins, heparin-affinity chromatography was used to first purify the total population of rFIX at greater than 90% yield. After the total population was isolated, the biologically active and inactive subpopulations were fractionated by high-resolution anion exchange chromatography using an ammonium acetate elution. Capillary isoelectric focusing of the active and inactive rFIX fractions demonstrated that the active subpopulations are the most acidic.
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Keywords: Milk; Tansgenic animals; Factor IX; Heparin; Proteins

1. Introduction

Hemophilia B is a bleeding disorder caused by a congenital deficiency in circulating factor IX activity. The ideal treatment of hemophilia B is prophylactic treatment, which significantly reduces complications and improves the quality of life [1]. Unfortunately, this regimen is unavailable for many hemophiliacs because the only sources of replacement factor IX are plasma-derived factor IX (pd-FIX) and recombinant factor IX (rFIX) produced in Chinese hamster ovary (CHO) cells (BeneFIX) [2]. Pathogen safety concerns with pd-FIX, combined with the limited supply of pd-FIX and rFIX from CHO cells make prophylactic treatment of hemophilia B expensive. Safer and more productive sources of rFIX are thus desired.

Factor IX is a complex protein with numerous post-translational modifications (PTMs). These PTMs are required for biological activity and pharmacokinetics, and include propeptide removal, vitamin K-dependent γ-carboxylation of Glu to γ-carboxyglutamate (Gla), Ser-phosphorylation, Tyr-sulfation, and glycosylation [3]. In particular, γ-carboxylation and propeptide removal are required for the procoagulant activity of factor IX (Fig. 1). The complexity of these multiple PTMs also necessitates production of rFIX in mammalian cells. However, not all mammalian cells are equally proficient in performing all of these PTMs. For example, CHO cells were shown to have significant rate limitations in propeptide removal, γ-carboxylation, and Ser-phosphorylation [4–6]. Additionally, transgenic sheep were not able to produce any significant amounts of biologically active rFIX in milk [7]. We have previously published immunoaffinity methods for purification of biologically active rFIX from transgenic pig milk having lower expression levels of 100–200 μg/ml containing 35–70 U/ml [8]. The rFIX was fully γ-carboxylated at this level of production.

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doi:10.1016/j.chroma.2003.11.006

Propeptide | Gla Domain

TVFLDHENANKILNRPKR (−1) | (+1) YNSGKLEEFVQGNLERECMEEKCSFEEAREVFENTERTTE

Fig. 1. Amino acid sequence of the factor IX propeptide and Gla domain. E represents potential γ-carboxyglutamate (Gla) residues (12 in total). The propeptide is cleaved prior to secretion.

Immunoaffinity chromatography was also used to purify biologically active rFIX from the milk of transgenic mice [9].

In this work, we report on the purification of rFIX from a new lineage of transgenic pigs having expression levels of 2–3 g rFIX/l milk containing about 70–140 U/ml FIX activity. With the ability to translate and secrete higher levels of recombinant polypeptide by transgenic methods comes the need for economical purification methods that can selectively isolate populations that have preferred post-translational structure. We demonstrate that non-immunoaffinity chromatographic methods are able to purify select rFIX populations to a high purity and a high specific activity. Importantly, we also demonstrate a rate limitation in γ-carboxylation occurs at rFIX expression levels of 0.2–0.3 g/l.

2. Experimental

2.1. Reagents

All buffer components were purchased from Sigma (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA), unless otherwise noted. The following buffers were used.

(i) Heparin loading buffer: 20 mM Tris, 50 mM NaCl, pH 7.2.
(ii) Heparin wash buffer: 20 mM Tris, 200 mM NaCl, pH 7.2.
(iii) Heparin elution buffer: 20 mM Tris, 500 mM NaCl, pH 7.2.
(iv) Heparin regeneration buffer: 4 M NaCl.
(v) Matrex Cellufine sulfate loading buffer: 20 mM Tris, 125 mM NaCl, pH 7.4.
(vi) Matrex Cellufine sulfate wash buffer: 20 mM Tris, 250 mM NaCl, pH 7.4.
(vii) Matrex Cellufine sulfate elution buffer: 20 mM Tris, 500 mM NaCl, pH 7.4.
(viii) *Q* loading buffer: 20 mM Tris, pH 9.0.

2.2. Transgenic milk collection and storage

Transgenic pigs were generated as described previously [8], but using a 4.2 kb mouse whey acid protein promoter for the factor IX cDNA construct [10]. Milk was collected from lactating pigs in accordance with protocols approved by the Virginia Tech Animal Care Committee. Lactating sows were injected intramuscularly with 60 units of oxytocin (Vedco, St. Joseph, MO), and milked by hand. The milk was immediately aliquoted and frozen at −50 °C. Milk used in this study had a factor X activity level of 100–140 U/ml milk, as measured by the activated partial thromboplastin time (APTT) assay.

2.3. Purification of rFIX

2.3.1. Heparin chromatography

Heparin-Sepharose FF (Amersham Biosciences, Piscataway, NJ, USA) was packed in Bio-Rad Econo-columns (Hercules, CA, USA) and the columns were run on a Bio-Rad BioLogic DuoFlow chromatography system with 280 nm detection. Aliquots of milk were thawed and mixed 1:1 (v:v) with 200 mM EDTA pH 7.4 (to dissolve the casein micelles) and centrifuged for 15 min at 3000 × *g* at 4 °C to separate the fat. The skim milk/EDTA was diluted five-fold with heparin loading buffer, and loaded onto the column (9 cm × 2.5 cm i.d.). All columns in this work were run at room temperature. In order to minimize rFIX losses in the flow-through and wash steps, the column was loaded to only 1 mg rFIX/ml matrix at a linear velocity of 1 cm/min. The column was subsequently washed with heparin loading buffer at 2 ml/min until the baseline absorbance was reached, and then washed with heparin wash buffer at 2 ml/min to remove adsorbed caseins. Recombinant factor IX was eluted with heparin elution buffer at 3 ml/min. The heparin product was either immediately diluted and processed by anion exchange chromatography, or else immediately frozen at −70 °C. The heparin-Sepharose column was regenerated with 4 M NaCl, and re-equilibrated.

A synthetic heparin analogue matrix, Matrex Cellufine sulfate (Millipore, Billerica, MA, USA), was also tested. The column was packed in a Bio-Rad Econo-Column (8 cm × 1 cm i.d.). The Cellufine sulfate column was first equilibrated with sulfate loading buffer and subsequently loaded at 1.5 ml/min with skim milk diluted identically as with the heparin-Sepharose columns. The column was then washed with Cellufine sulfate loading buffer until baseline was reached, and then washed using Cellufine sulfate wash buffer. Factor IX was eluted using Cellufine sulfate elution buffer. All steps were performed at 1.5 ml/min. As with heparin-Sepharose, the Cellufine sulfate column was regenerated with 4 M NaCl and re-equilibrated.

The purity of the heparin-Sepharose product was analyzed by reversed-phase HPLC on a Vydac 218TP5415 column (C_{18} stationary phase, 15 cm × 4.6 mM i.d., 5 μm particles, 300 Å pores, Hesperia, CA, USA) with a Supelco Supelguard LC318 guard column (C_{18}, 5 μm particles, 300 Å pores, St. Louis, MO, USA). The solvents used were: (A) 0.1% (w/v) trifluoroacetic acid (TFA) in water, and (B) 0.1% (w/v) TFA in acetonitrile. The heparin-

Sepharose product and immunoaffinity purified pd-FIX standard (Mononine, Centeon LLC, Kankakee, IL, USA) were diluted in deionized water and 200 μl were injected. The solvent program was as follows: 5 min, 5% B, followed by 5–95% B over 45 min (2%/min), followed by 5 min of 95% B. The flow rate was constant at 1 ml/min. Data were collected on a Beckman System Gold HPLC system with a Model 126 solvent delivery system and a Model 168 diode array detector. An Isco Foxy programmable fraction collector was used to collect fractions. Peak areas at 220 nm were integrated by the System Gold software.

2.4. Anion-exchange chromatography

A Mini Q 4.6/5.0 column (5 cm × 4.6 mM i.d. non-porous 3 μm particles, Amersham Biosciences) was used to fractionate active from inactive rFIX subpopulations. The heparin-Sepharose product was diluted with two parts *Q* loading buffer, and loaded at a flow rate of 0.4 ml/min. The column was washed with 90%:10% *Q* loading buffer:*Q* elution buffer for 10 column volumes (CVs) at 0.8 ml/min. Adsorbed rFIX was eluted by either a linear gradient or by a combination of gradient and isocratic elutions. Data were collected on the Beckman HPLC system. The specific conditions are given in the figure captions for the chromatograms shown.

2.5. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Column fractions were analyzed by SDS-PAGE (silver-stained) using Invitrogen (Carlsbad, CA, USA) gels and the Invitrogen Minicell apparatus. All gels were NuPage 10% bis-Tris run with 2-(*N*-morpholino)ethanesulfonic acid (MES) running buffer (Invitrogen). Briefly, samples were mixed with 4× LDS sample buffer (Invitrogen) and deionized water followed by heating at 95 °C for 10 min. For reduced gels, samples were adjusted to 0.05 M dithiothreitol (DTT) prior to heating.

2.6. Factor IX activity—APTT assay

The biological activity of the rFIX fractions was determined by the APTT assay [11]. Briefly, 100 μl each of Alexin (cephalin from rabbit brain, Sigma), factor IX deficient plasma (Biomerieux, Durham, NC, USA), and sample of interest were added to a cuvette and incubated at 37 °C for 3 min. One hundred microlitres of 20 mM $CaCl_2$ (Sigma) was then added and the clotting time was measured using a MLA Electra Coag-a-Mate coagulation timer. Units of factor IX activity were calculated by comparison of the unknown sample to a standard curve created from dilutions of normal reference plasma (Biomerieux, 1 U/ml plasma) made using the same protocol. The concentration of purified rFIX was determined by measuring the optical density at 280 nm in a 1 cm quartz cuvette and using an extinction coefficient of 1.33 for a 1 mg/ml factor IX solution.

2.7. Total Gla analysis

Total Gla content of the purified rFIX fractions was determined by amino acid analysis from basic hydrolysis. Column fractions were dialyzed overnight against deionized water in a cold room (4 °C) and then lyophilized to dryness in a Savant Speed-Vac system. The rFIX was then reconstituted in deionized water, frozen, and shipped to Commonwealth Biotechnologies (Richmond, VA, USA) for analysis. Briefly, 83 μl of 4 M NaOH and 117 μl of water were added to 100 μl of sample. The tubes were sealed under vacuum and hydrolyzed for 20 h at 100 °C. The samples were then neutralized with 4 M acetic acid, diluted with a borate buffer, and then subjected to analysis. The molar ratio $R = \text{Glu}/\text{Gla}$ for each sample was obtained from the respective peak areas of the chromatogram. Factor IX has 40 total Glx residues (28 Glu + 12 potential Gla). The (moles Gla)/(moles factor IX) was obtained by solving the simple set of equations:

$$\text{Glu} + \text{Gla} = 40$$

$$\frac{\text{Glu}}{\text{Gla}} = R$$

where R is the experimentally determined ratio of (moles of Glu)/(moles of Gla) for the sample.

2.8. Isoelectric focusing

The isoelectric point (p*I*) of rFIX subpopulations was determined by a two-step capillary isoelectric focusing and mobilization based on the method reported by Huang et al. [12]. A two step method was necessary because factor IX is a very acidic protein. Polyacrylamide coated capillaries (eCAP Neutral, Beckman Coulter, Fullerton, CA, USA) of 27 cm total length × 50 μm i.d. were used with a Beckman PACE/5000 instrument with UV absorbance detection. The anolyte used was 60 mM phosphoric acid in 0.4% (w/v) hydroxypropylmethylcellulose (HPMC, Sigma). The catholyte was 20 mM NaOH. All solutions were filtered with a 0.2 μm syringe filter before use. Ampholytes (p*I* 3–10) were purchased from Fluka (St. Louis, MO, USA). Two standards, carbonic anhydrase I (Sigma, pI = 6.6) and amyloglucosidase (Sigma, pI = 3.6) were included in all focusing experiments. The sample solution was prepared by combining the factor IX sample, the two protein standards (2 μl of 4 mg/ml carbonic anhydrase I and 1 μl of 4 mg/ml amyloglucosidase), ampholytes (1 μl), and 1% HPMC (80 μl, for final 0.4% HPMC) to a total volume of 200 μl. The capillary was first rinsed with deionized water for 2 min and then filled with the sample solution by rinse mode (20 psi; 1 psi = 6894.76 Pa) for 1 min. Normal polarity (cathode at detector end) was used for focusing and mobilization. Focusing was performed by applying 15 kV for 6 min. After the focusing step, a low pressure rinse of anolyte (0.5 psi) while maintaining 15 kV was applied for 50 min to mobilize the focused proteins past the detector window. The separations were car-

ried out at 20 °C with detection at 280 nm. The capillary was rinsed with deionized water for 5 min after each run.

3. Results and discussion

Efforts to purify factor IX from donor plasma are decades old, and production of factor IX concentrates by crude methods such as cryoprecipitation or Cohn fractionation has been replaced with high purity products using immunoaffinity and heparin-affinity chromatography [13–15]. Milk and blood are equally challenging feedstocks for protein purification as both are multi-phase mixtures. Milk has a solids phase that includes cell debris and the casein micelles, an aqueous phase that contains whey proteins, carbohydrates, minerals and nutrients, and the milkfat phase that contains triglycerides, phospholipids, and cholesterol [16]. The caseins are the most prevalent endogenous milk proteins at about 30 g/l. Other major milk proteins include whey proteins (lactoferrin, α-lactalbumin, β-lactoglobulin) and serum-passover proteins (serum albumin, immunoglobulins, transferrin). Even though rFIX is being produced at a very high level of 2–3 g/l in the milk, it is still less than 10% of the total protein content. In addition, the numerous variations in the PTMs that are made to this complex protein add another layer of complexity to the purification problem—how does one design a purification process that also selects for only the biologically active subpopulations?

The initial objective was to purify the entire population of rFIX from the milk so as to understand the nature of limits in post-translational processing of rFIX that occurs at these very high expression levels. The use of classical casein precipitation techniques was problematic, with as much as 20% of the rFIX partitioning into casein precipitates (data not shown). Thus, to circumvent this problem, the casein micelles were solubilized with excess EDTA. The heparin binding domain of factor IX is located in the C-terminal end of the molecule, a region devoid of PTMs [17]. We therefore used heparin-affinity chromatography as a capture step so that the entire population could be isolated. The rFIX was found to strongly bind to the heparin matrix. The column was underloaded (with respect to factor IX capacity) to result in complete rFIX capture. Caseins as well as a number of other milk proteins also adsorbed to the column, but were washed off with 200 mM NaCl (Fig. 2). Highly pure rFIX was eluted with 500 mM NaCl. The purity of the rFIX product was analyzed by reversed phase HPLC. Using TFA as an ion pairing agent and a 2%/min acetonitrile gradient, the isoforms of both plasma-derived factor IX and rFIX were not resolved, but eluted as a single peak (Fig. 3). The purity from this single chromatographic step (98% peak area at 220 nm) was similar to the purity of pd-FIX purified by immunoaffinity chromatography. Western blots and activity assays of the column flow-through and 200 mM NaCl fractions had undetectable rFIX antigen and activity.

A synthetic heparin analogue, Matrex Cellufine sulfate, has also been used in the purification of BeneFIX, a rFIX produced in CHO cells [18]. We were unable to obtain a pure rFIX fraction with this heparin analogue when clarified skim milk was used as the column feed (Fig. 4). Washing the column with a 250 mM NaCl buffer resulted in the loss of rFIX, and a significant amount of the caseins co-eluted with rFIX in a 500 mM NaCl buffer. In fact, caseins remained adsorbed to the column and had to be removed during the regeneration step with 4 M NaCl. The data show that the heparin analogue did not have the specificity to discrimi-

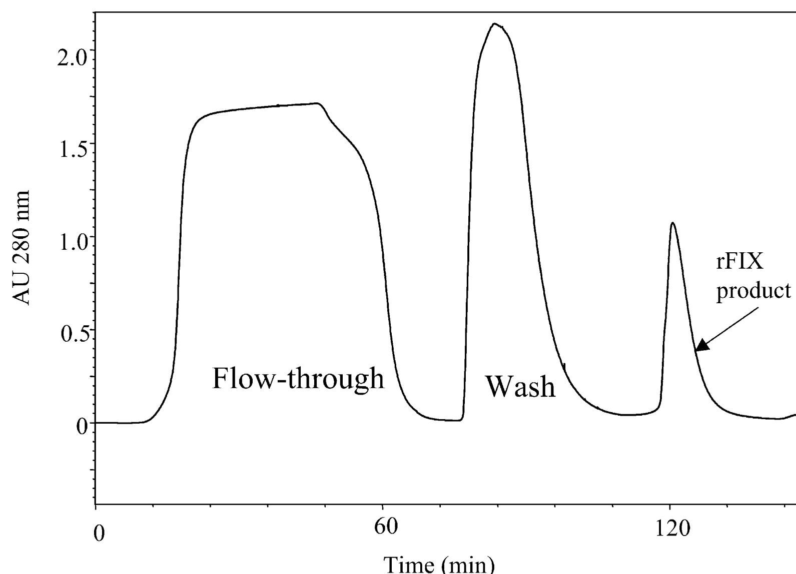

Fig. 2. Purification of rFIX from transgenic pig milk by heparin-affinity chromatography on heparin-Sepharose. The feed to the column contained 11 ml of milk that was diluted with EDTA, centrifuged, and diluted in column loading buffer. The column was washed with 20 mM Tris, 200 mM NaCl, pH 7.2 to remove non-specifically bound milk proteins. Pure rFIX was eluted with 20 mM Tris, 500 mM NaCl, pH 7.2; 31 mg of rFIX were recovered in the elution peak.

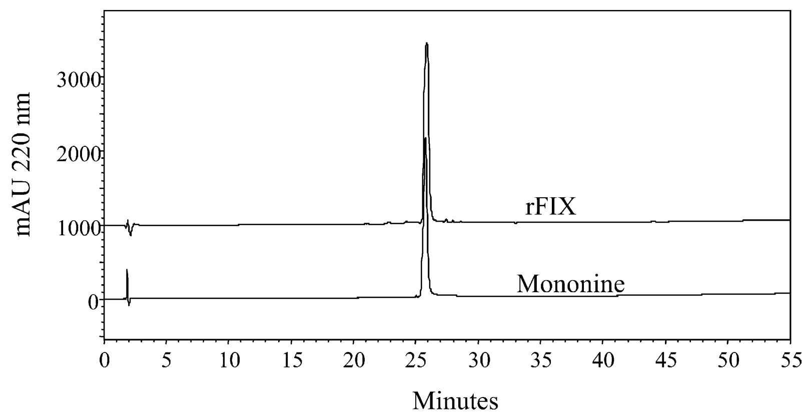

Fig. 3. HPLC analysis of the purity of the heparin-Sepharose product: 0.35 mg/ml; 100 μl injected. For comparison, an immunoaffinity purified plasma-derived factor IX (Mononine): 0.5 mg/ml, 50 μl injected. Column: Vydac C_{18}, 15 cm × 4.6 mM. Solvents: (A) 0.1% (w/v) TFA in water, and (B) 0.1% (w/v) TFA in acetonitrile. Flow rate: 1 ml/min. Solvent program: 5 min, 5% B, followed by 5–95% B over 45 min, followed by 5 min of 95% B.

nate between rFIX and the caseins, and non-specific ion exchange interactions with the sulfated cellulose matrix were likely dominating for the conditions used. Thus, unless the caseins are removed, this heparin analogue is not useful for purification of heparin-binding proteins from porcine milk.

The specific activity of the heparin product was low (typically 30–35 U/mg protein) compared to the theoretical pd-FIX specific activity of 200–250 U/mg. This indicated that only a fraction of the total rFIX population was biologically active. The biological activity of factor IX is highly dependent on the nature and extent of PTMs. Of primary importance are the PTMs that are made to the N-terminal region of factor IX—the Gla domain. For factor IX to be active, the propeptide must be removed, and the Glu residues in the Gla domain must be γ-carboxylated [3]. The conformation of a fully γ-carboxylated Gla domain undergoes a dramatic change in the presence of Ca^{2+} [19,20]. This Ca^{2+}-dependent conformational change enables localization

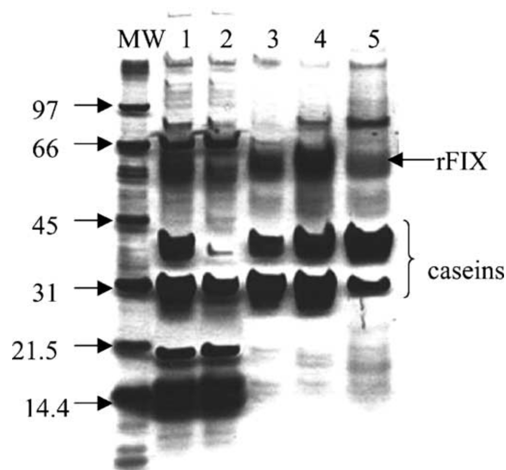

Fig. 4. Silver-stained SDS-PAGE of column fractions from Matrex Cellufine sulfate, a synthetic heparin analogue. Lane 1: column feed (skim milk/EDTA); lane 2: column flow-through and wash; lane 3: 250 mM NaCl eluate; lane 4: 500 mM NaCl eluate; lane 5: 4 M NaCl eluate. MW: molecular mass, scale in kilodaltons.

of factor IX on the endothelial or platelet cell surface, where it associates with factor VIIIa to activate factor X. Inadequate γ-carboxylation or the presence of propeptide severely diminishes this metal-dependent conformational change and renders the factor IX inactive [21,22].

Several different methods have been used to fractionate highly γ-carboxylated, active rFIX from inactive rFIX, and they are all based on the metal-dependent conformational changes of the Gla domain. Barium citrate adsorption was one of the earliest methods used [23], but this has the disadvantage of long processing times and is not easily scaled up. More recently, metal-dependent monoclonal antibodies have been raised against the Gla domain [24]. Other pseudo-affinity chromatographic methods have also been developed: Yan et al. used a calcium elution off an anion exchange resin to fractionate fully γ-carboxylated recombinant Protein C from uncarboxylated subpopulations [25]. This method, as well as hydroxyapatite chromatography, has also been adapted for BeneFIX production [18]. For the rFIX heparin chromatography product from transgenic pigs, hydroxyapatite chromatography and the pseudo-affinity calcium elution off an anion exchange column did not cleanly fractionate active from inactive subpopulations. Barium citrate adsorption was moderately successful in fractionating active from inactive subpopulations, but the specific activity of the pellet fraction was low (∼50 U/mg) due to the fact that propeptide-containing rFIX also partitions into the pellet fraction [26,27], which was confirmed by N-terminal amino acid sequencing (data not shown).

γ-Carboxylated proteins are highly acidic and this favors the use of anion exchange to obtain the most highly biologically active rFIX species. We adapted the method of Gillis et al. [28], which used a NaCl gradient elution from a high-resolution anion-exchange column to partially resolve rFIX made in CHO cells having 10, 11, or 12 Gla residues. The chromatogram from a NaCl gradient elution of rFIX on a Mini Q column is given in Fig. 5A. The factor IX begins eluting at about 0.2 M NaCl, and multiple subpopulations elute as the salt concentration is increased at a rate of 5 mM

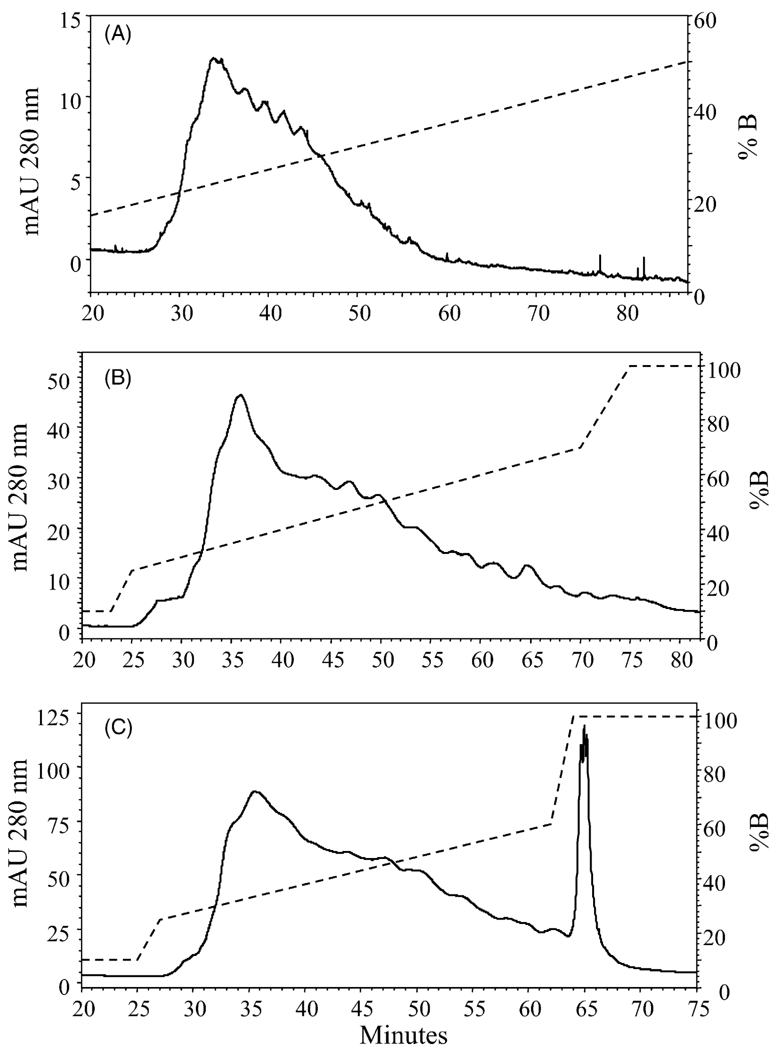

Fig. 5. Anion-exchange chromatography of heparin-purified rFIX on a Mini Q column. (A) NaCl gradient elution. Buffer A: 20 mM Tris, pH 9.0. Buffer B: 20 mM Tris, 1 M NaCl, pH 9.0. Load rFIX at 0.4 ml/min at 10% B. Wash with 10% B at 0.8 ml/min. Elute with linear gradient of 10–50% B over 80 min ($t = 7$–87 min). Loaded 0.3 mg rFIX. (B) Ammonium acetate gradient elution. Buffer A: 20 mM Tris, pH 9.0. Buffer B: 20 mM Tris, 1 M ammonium acetate pH 9.0. Load rFIX at 0.4 ml/min at 10% B. Wash with 10% B at 0.8 ml/min. Elution program: 10–25% B over 2 min, 25–70% B over 45 min, 70–100% B over 5 min, 100% B for 5 min. Loaded 0.6 mg rFIX. (C) Modified ammonium acetate gradient elution. Elution program: 10–25% B over 2 min, 25–60% B over 35 min, 70–100% B over 2 min, 100% B for 10 min. Loaded 1.6 mg rFIX.

NaCl/CV. Analysis of the fractions showed that the biologically active subpopulations eluted in the tail end of the peak that corresponded to about 10% of the peak area. However, even with this high-resolution column matrix there is poor resolution between the subpopulations in this region, and low specific activities of collected fractions indicated that subpopulations with inadequate Gla content and/or pro-factor IX were not well resolved. Resolution was not significantly improved by using a gradient slope of 2.5 mM NaCl/CV, and resolution was even poorer at higher rFIX loading.

In an effort to obtain better resolution between the active and inactive subpopulations, a different displacing salt was tried. Kopaciewicz et al. showed that the resolution between soybean trypsin inhibitor and ovalbumin on a strong anion exchange column was dependent on the counterion [29]. We therefore used an elution buffer with ammonium acetate for gradient elution of rFIX. The results of an ammonium acetate gradient on the same Mini Q column are shown in Fig. 5B. Again, the biologically active rFIX eluted in the last 10% of the peak area (beginning at 61 min). However, the use of ammonium acetate resulted in better resolution between subpopulations, especially in the later eluting subpopulations where biologically active rFIX elutes. There are several factors that are likely contributing to this result. The acetate counterion is known to have a weaker elution strength than chloride, and the ammonium ion is more chaotropic than the sodium ion. Additionally, in a detailed study of the effect of salt counterion on protein retention in anion exchange chromatography, Malmquist and Lundell concluded that non-specific effects could play an important role also [30]. A more detailed mechanistic explanation of the cause of this behavior is beyond the current scope of

this work, but the data show that the salt species can play a significant role in improving the resolution between closely related subpopulations of the same protein.

The elution program was then modified to obtain a more concentrated product (Fig. 5C). The active rFIX subpopulations were eluted in the triplet peak obtained after the sharp increase in ammonium acetate from 0.6 to 1.0 M, and corresponded to 10% of the total elution peak area. The overall Gla content of a pool of fractions of 63–67 min from six replicate runs was measured by amino acid analysis after basic hydrolysis, and was determined to be 6.0 mol Gla/mol rFIX. Parallel analysis of Mononine gave a result of 10.5 moles Gla/mole factor IX (theoretical value is 12 moles Gla/mole factor IX). Our results are similar to rFIX that was produced in CHO cells, where the average Gla content was 6.5 moles Gla/mol rFIX [5], and again demonstrate that γ-carboxylation of *all* 12 Glu residues in the Gla domain is not a necessity for biological activity.

The results from the Gla analysis also revealed why previously published factor IX purification techniques were unsuccessful in fractionating active from inactive subpopulations. It is known that *human* γ-glutamyl carboxylase modifies human factor IX by a tethered-processive mechanism [31,32]. That is, the carboxylase binds to the factor IX propeptide and sequentially modifies all Glu residues in the Gla domain during that single binding event. The unmodified Glu residues in rFIX produced in the pig are most likely at the C-terminal end of the Gla domain. Zhang et al.

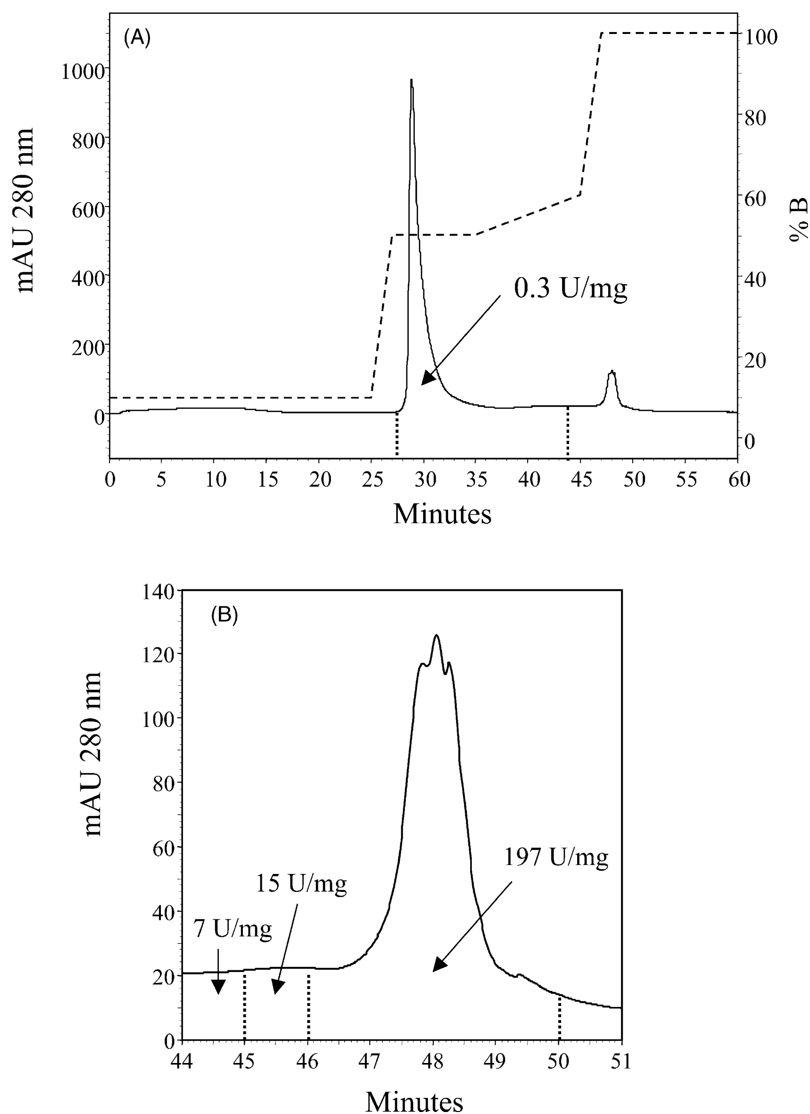

Fig. 6. Anion-exchange fractionation of biologically active and inactive subpopulations of rFIX on a Mini Q column. Loaded 1.6 mg rFIX. (A) Purified rFIX from the heparin-Sepharose column was diluted three-fold with *Q* loading buffer and loaded at 0.4 ml/min. After washing with 0.1 M ammonium acetate, the inactive rFIX was eluted by a sharp increase to 0.5 M ammonium acetate over a 2 min period. After holding at 0.5 M ammonium acetate for 8 min, the ammonium acetate was increased to 0.6 M over a period of 10 min. The biologically active rFIX was then eluted by a sharp increase to 1.0 M ammonium acetate. The effluent from 27 to 44 min was pooled. (B) An expanded view of the biologically active rFIX elution peak, along with the specific activity of the fractions as determined by the APTT assay. Duplicates of two dilutions of each fraction were submitted to the APTT assay.

found that conserved mutations of Glu to Asp in the Gla domain of Protein C (a protein with a homologous Gla domain) did not necessarily abolish biological activity, but did affect the Ca^{2+}-dependent conformational changes [33,34]. Therefore, subtle differences in the Ca^{2+}-dependent conformation of rFIX caused by *partial* γ-carboxylation are likely the reason why hydroxyapatite and Ca^{2+}-specific pseudo-affinity fractionation methods were not successful for rFIX produced at these very high expression levels.

The gradient elution depicted in Fig. 5C was further modified to obtain a shorter run time and be more amenable for future scale-up. As shown in Fig. 6A, the majority of the inactive rFIX subpopulations were eluted by a sharp increase to 0.5 M ammonium acetate. The specific activity in the pooled fraction from 27 to 44 min was 0.3 U/mg. The expanded view of the elution peak is given in Fig. 6B. As the ammonium acetate was slowly increased up to 0.6 M, rFIX subpopulations with low specific activity began to elute. rFIX with high activity was then eluted by a sharp increase to 1 M ammonium acetate. Again, a characteristic triplet peak was obtained, and the area percentage of this elution peak (46–50 min) was approximately 10% of the total peak area of the chromatogram for all replicate runs. The average specific activity of the fraction from 46–50 min from a representative isolation was 197 U/mg. This translates to a net amount of about 0.3 g/l rFIX that is biologically active. Thus, this level of active rFIX production is similar to the previously reported lineage of transgenic pigs producing 100% biologically active rFIX at a total expression level of 0.2 g/l, and it indicates that a rate limitation in γ-carboxylation occurs in the range of 0.2–0.3 g/l.

The anion exchange column with ammonium acetate elution method is highly reproducible, and results in a cleaner fractionation of active subpopulations compared to a NaCl elution. Isoelectric focusing of the products confirmed that the most active rFIX subpopulations that are fractionated by anion exchange chromatography are also the most acidic (Fig. 7). However, the biologically active rFIX produced by the transgenic pig is less acidic than plasma-derived factor IX. This is at least partially due to the fact that overall Gla content of the entire 46–50 min elution fraction was lower than pd-FIX. This lower acidity may also be due to differences in sialic acid content, although Bharadwaj et al. demonstrated that removal of sialic acid had no effect on factor IX activity [35]. Further characterization of glycosylation, phosphorylation, and sulfation made to rFIX subpopulations produced in the transgenic pig is in progress.

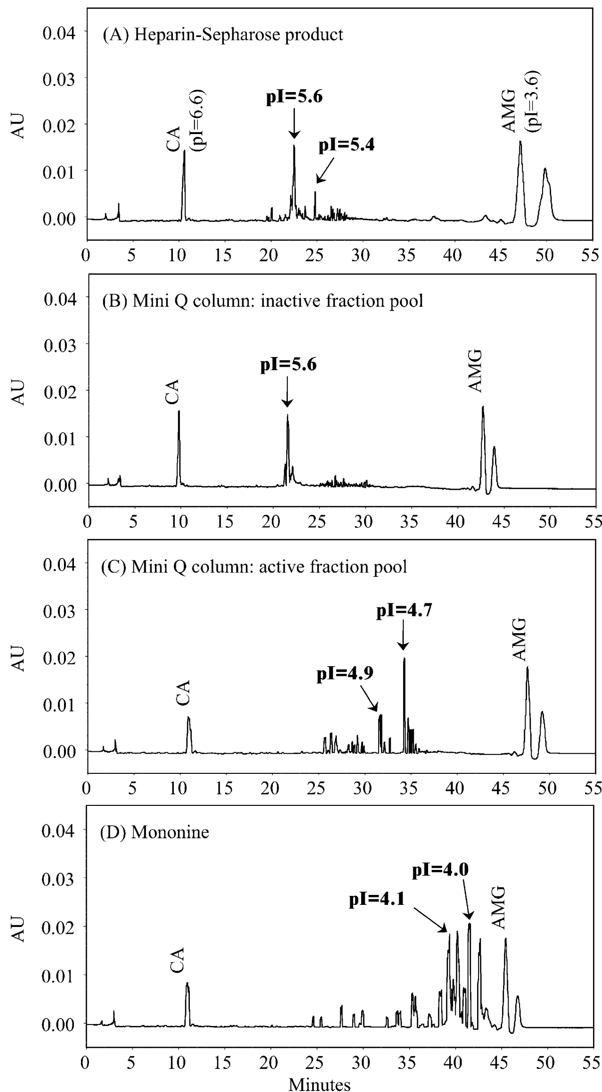

Fig. 7. Capillary isoelectric focusing electropherograms of rFIX subpopulations and plasma-derived factor IX. (A) 40 μg/ml of rFIX purified by heparin-Shepharose, (B) 30 μg/ml of rFIX-inactive fraction pool from Mini Q column, $t = 27$–44 min, (C) 50 μg/ml of rFIX-active fraction pool from Mini Q column, $t = 46$–50 min, (D) 40 μg/ml of Mononine-immunoaffinity purified plasma-derived factor IX. Estimates of the p*I* of major rFIX and Mononine subpopulations are given using a linear interpolation of p*I* vs. time for the two standards: carbonic anhydrase I (CA, $pI = 6.6$) and amyloglucosidase (AMG, $pI = 3.6$).

4. Conclusions

The purification of complex recombinant proteins is complicated by the fact that multiple subpopulations corresponding to differences in post-translational processing are usually present. In many cases, the PTMs have a significant impact on the biological activity and pharmacokinetics, and so the purification process will need to select for the best subpopulations. Heparin-affinity chromatography was found to be an ideal method for purifying the total rFIX population in a single chromatographic step. However, a synthetic heparin analogue, Matrex Cellufine sulfate, did not have the specificity required for purification of a heparin-binding protein from milk. Because the rFIX produced at expression levels of 2–3 g/l was partially γ-carboxylated, conventional chromatographic techniques that have been used in the past for fractionation of active from inactive factor IX subpopulations were not efficient. The heparin-chromatography product was fractionated into active and inactive subpopulations

by high-resolution anion-exchange chromatography. Changing the salt displacer from NaCl to ammonium acetate enabled us to more cleanly resolve the biologically active and inactive rFIX subpopulations. In vitro coagulation activity assays and capillary isoelectric focusing confirmed that the most acidic subpopulations that eluted at higher acetate concentrations were the most active, and accounted for about 10% of the rFIX that is being produced in the milk at an overall expression level of 2–3 g/l. Thus, a highly pure and biologically active rFIX product was obtained from transgenic animal milk without the use of immunoaffinity chromatography.

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