boratory of
mmunobiochemistry

Operational issues

B - missions

- *Research*
- *Product quality*
- *Regulatory support*
- *Industry support*

ting chiefs of LIB, 1997-98

- *Paul Turkeltaub, MD*
- *Richard Pastor, PhD*

B staffing

- *Full time*
  - *Jay E. Slater, MD - Lab Chief*
  - *Lyudmila Soldatova, PhD - ORISE*
  - *Maneesha Solanki - Biologist*
  - *Elizabeth Paupore - Biologist*
- *Part time*
  - *Al Gam - Biologist*
  - *Gerald Poley, MD - Guest Worker*
  - *Li-Shan Hsieh, PhD - CDER*

Routine" regulatory activities

- *Protocol review*
- *Product testing*
- *Reference development*
- *Reference distribution*
- *Reference maintenance*
  - *semiannual checks*
  - *replacement*

ecific projects - ELISA timization

- *Objective: critical re-evaluation of the method*
- *Validate to new assay*

ELISA optimization

- *Buffer detergent has been changed from Brij to Tween-20.*
- *The blocking buffer and test diluent have been changed to PBS pH 7.4 containing 0.05% Tween-20 and 1.0 % bovine serum albumin*
- *Coating, competition and conjugate incubations are all overnight.*
- *TMB substrate is equilibrated at room temperature for 5 min; TMB incubation step is exactly 5 min.*

ELISA validation results

- *No 1.0 extracts failed*
- *No 0.5/2.0 extracts passed*
- *SD within old limits (0.1375)*
- *Accurate with all three antigens, at full range of concentrations*

		N	lp	sd(log lp)
meadow	0.5	24	0.516	0.097
fescue	1	24	1.104	0.107
	2	24	2.085	0.152
D. farinae	0.5	23	0.499	0.109
	1	23	1.067	0.105
	2	23	2.19	0.113
Bermuda	0.5	23	0.464	0.116
	1	23	0.9914	0.102
	2	23	1.94	0.125

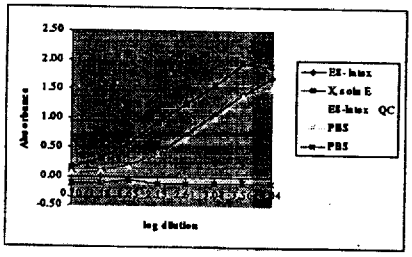
New reference sera/extracts

- *Cat S2 replaced by S2a*
- *Mite S3 replaced by S4*
- *Latex S2 replaced by S3*
- *Dp and cat extract replacements in progress*

ecific projects - new latex rum pool

- Prepared 12/98
- Pooled from seven adults with latex allergy
  - All bands in E8 detected

ecific projects - new latex rum pool



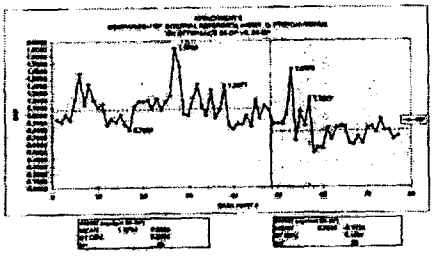
ecific projects - new latex rum pool

	X, 1 mg/mL	E8, 3.9 mg/mL
Mean log RP	-0.63	0.04
SD (log RP)	0.05	0.05
Mean RP	**0.24**	**1.10**
95% CI	**0.19 to 0.30**	**0.89 to 1.35**

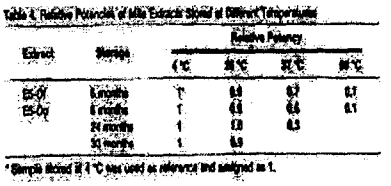
ite stability - the issues

- Cysteine and serine protease activity of mite antigens
- Conflicting prior data on stability
  - Nelson et al. J Allergy Clin Immunol 1996;98:382.
  - Liu and Lin. Ann Allergy Asthma Immunol 1998;80:177.
- Problems associated with short shelf life of reference materials

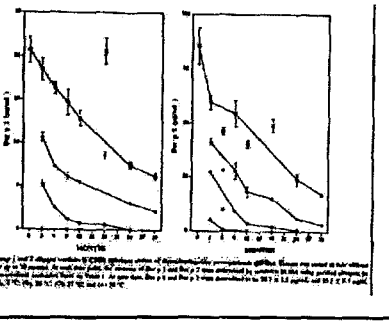
ite stability - the issues



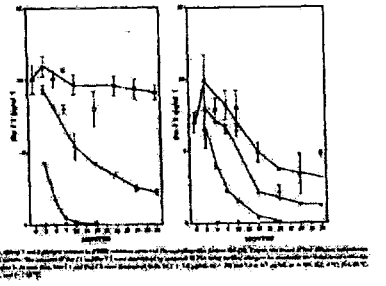
u and Lin, 1998



u and Lin, 1998



u and Lin, 1998



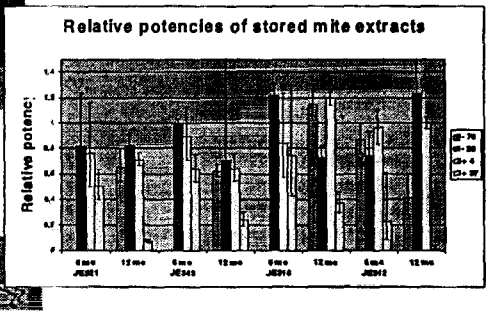
u and Lin, 1998 -conclusions d further questions

- *Der p 1, Der p 2 and Der f 2 unstable at 4°C*
- *Der f 1 stable at 4°C, unstable >26°C*
- *RP conserved at 26°C relative to 4°C*
- *Can protease inhibitors slow the degradation?*
- *Is RP conserved in the 4°C specimen relative to lyophilized?*

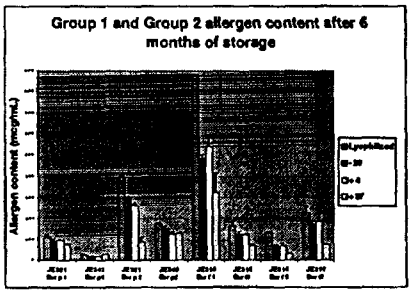
ite stability - experimental sign

- *Objective: identify and characterize possible degradation in glycerinated mite extracts, with and without protease inhibitors*
- *Glycerinated extracts stored at -70°C, -20°C, +4°C and +37°C for 6-12 months*
- *Compare to lyophilized standard*
- *Assayed by three methods*
  - *competitive ELISA for relative potency*
  - *two-site ELISA for group 1 and group 2 antigens*
  - *Western blot (sera and monoclonals)*

ite stability study



ite stability study



ite stability study - tentative nclusions

- *RP stable at 4°C relative to lyophilized*
- *loss of protein bands at 4°C*
- *loss of specific mite allergens at 4°C; this does not correlate with RP*
- *protease inhibitors offer no long-term protection*

ference replacement program

Current references:

- *many out of date (20/24)*

Replacement program:

- *bring full inventory up to date*
- *target completion date: August 2001*

ference replacement program

- *Proactive - candidates will be identified >6 months prior to expiration*
- *Comprehensive - all reference materials will be updated*
- *Anticipated problems - frequent replacement required*
  - *possible solutions : lyophilized references; or ELISA based on serum pools*

ference replacement program - oposal

- *1998-99 : CBER will lyophilize a portion of all reference extracts/sera*
- *1999-00 : CBER will assess stability and reliability of lyophilized products*
- *Results and samples will be distributed to APMA membership prior to action*

ues for long term nsideration

- *Should CBER continue to be the source of reference standard allergens and antisera?*
- *How should the standardization program proceed?*

BER's role as source of US placement reference materials

- *LIB identifies candidate reference*
  - *in house testing*
  - *samples sent to manufacturers for testing*
- *LIB purchases 1-3 year supply*
- *LIB distributes to manufacturers*

BER's role as source of US ference materials

- *Advantages*
  - *Control*
  - *Monitoring*
  - *Fairness*
  - *Available to investigators*
- *Disadvantages*
  - *Inventory management*
  - *Cost*

BER's role as source of US ference materials - current status

- *CBER will continue in its current role*
- *LIB will upgrade the reference stocks and evaluate better methods of maintaining the inventory*

ndardization - current radigm

- *Products are heterogeneous*
- *Products are natural*
  - *glycosylated*
  - *intact proteins*
- *Correlation between allergenicity and immunomodulatory activity*

rrent standardization targets

- *Latex*
- *Cockroach*
- *Tree pollens*

evea latex antigens

- *Hev b 1 (REF)*
- *Hev b 2 (β-1,3-glucanase)*
- *Hev b 3 (microhelix component)*
- *Hev b 4*
- *Hev b 5*
- *Hev b 6 (prohevein)*
- *ev b 7 (patatin nalogue)*
- *hevamine*
- *chitinases (I and II)*
- *Mn-superoxide dismutase*
- *enolase*
- *profilin*
- *lysozyme*
- *proteasome subunit*

ndardization - limitations

- *Uncertain predictive value for*
  - *peptides*
  - *plasmids*
  - *modified allergens*
  - *non-glycosylated products*
- *Cost borne by FDA*

oned products are inevitable
nunotherapy doses are 10-30 µg/month)

Product	Source	Size	Cost
Filgrastim	E. coli	175 aa	$0.53/µg
Sargramostim	Yeast	127 aa	$0.52/µg
Epoetin-α	Chinese hamster ovary	165 aa	$0.012/U =$1.55/µg

ndardization - alternative proaches

- *1. Consistency monitoring*
- *2. Pure allergen basis (monoclonal antibody)*
- *3. Other in vitro characterization*

- *Disadvantages*
- *No industry standard*
- *All allergens not identified or characterized*
- *Criteria not established*

ndardization - current ogram

- *completion of latex standardization within 6 months*
- *initiate work on cockroach standardization*

B staffing

- *Current*
  - *Lab Chief*
  - *Post-doc*
  - *Two biologists*
  - *One new biologist has been hired (to start 3/1/99)*
- *Proposed*
  - *Add one more biologist*

boratory of mmunobiochemistry

Research report

B research program summary

- *Allergen structure and function*
- *Immunomodulation*
- *Glycosylation*
- *Enzyme activity*
- *Identification methods*
- *Epitopes*
- *DNA vaccines*
- *LPS*
- *Cross-sensitization*

blications from LIB staff, 98-1999

- Liu T., Lin Y. (1998) The epitope stability of Group 1 and Group 2 allergens in mite extracts. *Ann Allergy Asthma Immunol* **80**, 177-183.
- Soldatova L.N., Crameri R., Gmachl M., Kemeny D.M., Schmidt M., Weber M. and Mueller U.R. (1998) Superior biologic activity of the recombinant bee venom allergen hyaluronidase expressed in baculovirus-infected insect cells as compared with Escherichia coli. *J Allergy Clin Immunol* **101**, 691-698.
- Sowka S., Hsieh L.S., Krebitz M., Akasawa A., Martin B.M., Starrett D., Peterbauer C.K., Scheiner O. and Breiteneder H. (1998) Identification and cloning of prs a 1, a 32-kDa endochitinase and major allergen of avocado, and its expression in the yeast Pichia pastoris. *J Biol Chem* **273**, 28091-28097.

blications from LIB staff, 98-1999

- **Slater J.E., Paupore E.,** Zhang Y.T. and Colberg-Poley A.M. (1998) The latex allergen Hev b 5 transcript is widely distributed after subcutaneous injection in BALB/c mice of its DNA vaccine. *J Allergy Clin Immunol* **102**, 469-475.
- **Slater J.E., Paupore E.J.,** Elwell M.R. and Truscott W. (1998) Lipopolysaccharide augments IgG and IgE responses of mice to the latex allergen Hev b 5. *J Allergy Clin Immunol* **102**, 977-983.
- **Slater J.E., Paupore E.J.** and O'Hehir R.E. (1999) Murine B-cell and T-cell epitopes of the allergen Hev b 5 from natural rubber latex. *Molecular Immunology* (In Press)

lergen structure and function glycosylation

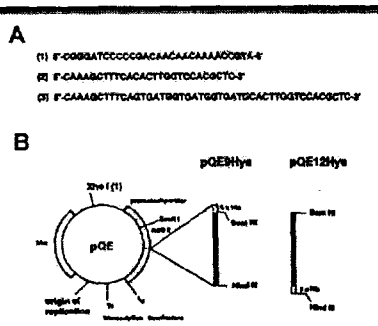
- *Is the decreased antibody binding of non-glycosylated antigens primarily a function of impaired folding?*
- *What is the biochemical anatomy of the glycosylation requirement?*
- *Can non-glycosylated allergens equal native allergens in immunotherapy?*
- *How can non-glycosylated products be evaluated for diagnosis and therapy?*

lergen structure and function enzyme activity

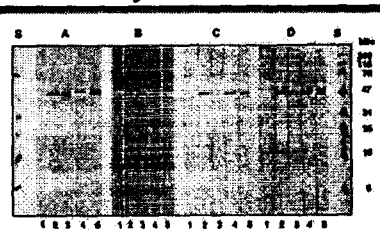
- *What is the relationship between enzyme activity and allergenicity?*
  - *antibody binding*
    - *in vitro*
  - *bioavailability*
  - *antigen processing*
- *Specific regulatory applications*
  - *hymenoptera, mites, latex*

atova L.N., Crameri R., Gmachl M., Kemeny ., Schmidt M., Weber M. and Mueller U.R. 8). Superior biologic activity of the ecombinant bee venom allergen hyaluronidase expressed in baculovirus-infected insect cells as compared with Escherichia coli. *J Allergy Clin Immunol* **101**, 691-698.

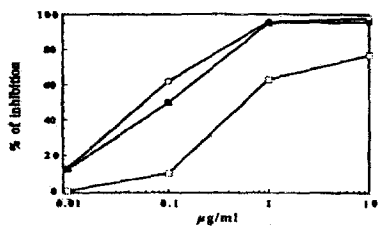
oning and expression of aluronidase



mmunoblot of natural and combinant hyaluronidases



ST inhibition with hyaluronidases



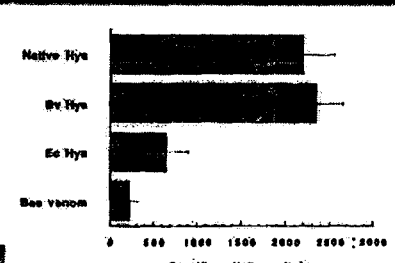
- *Open circles: nHya; filled squares: BvHya; open squares, EcHya*

rect RAST

TABLE I. Specific IgE against nHya, BvHya, and EcHya in 20 patients allergic to bee venom and 20 nonallergic control subjects

	RAST class	Specific IgE to (%)		
		nHya	BvHya	EcHya
Patients (n = 20)	0	1 (5)	1 (5)	8 (40)
	1	0	2 (10)	2 (10)
	2	4 (20)	5 (25)	7 (35)
	3	11 (55)	9 (45)	3 (15)
	4	4 (20)	3 (15)	0
Control subjects (n = 20)	0	20	20	20

ecific enzymatic activity of natural d recombinant hyaluronidases



luronidase expressed in baculovirus-cted insect cells - conclusions

- Honeybee hyaluronidase was expressed
- For enzyme activity: N = Bv > Ec
- For IgE binding: N = Bv > Ec

maining bee venom allergens

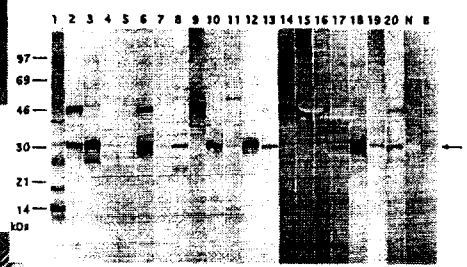
Acid phosphatase

- cloned from cDNA using primers determined from a genomic sequence
- about half of the putative sequence identified
- strong homology to insect ap, none to mammalian or leishmania ap

Allergen C

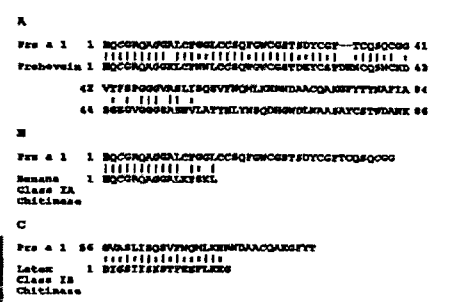
yka S., Hsieh L.S., Krebitz M., Akasawa A., rtin B.M., Starrett D., Peterbauer C.K., Scheiner nd Breiteneder H. (1998) Identification and ning of prs a 1, a 32-kDa endochitinase and major allergen of avocado, and its expression in the yeast Pichia pastoris *J Biol Chem* 273, 28091-28097.

E binding to avocado extracts

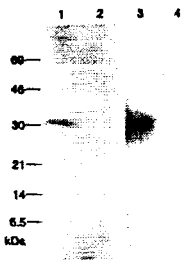


rs a 1 and rPrs a 1 (SDS-PAGE)

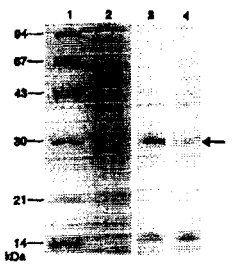
quence homologies of Prs a 1



E binding to rPrs a 1



hibition of IgE binding to ocado extracts by rPrs a 1



ntification and cloning of Prs a 1, a major rgen of avocado - conclusions

- *Prs a 1 was cloned and sequenced*
- *IgE binding: natural = recombinant*
- *Endochitinase activity*
- *Fungicidal activity*

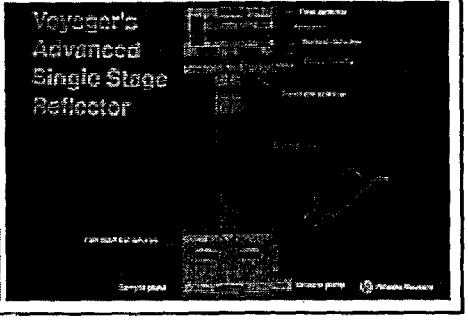
lergen structure and function - ditional questions

- *If an allergen that is not glycosylated, glycosylated abnormally, or denatured shows poor IgE binding or impaired enzymatic activity, how can we evaluate its efficacy as an immunotherapeutic reagent?*

lergen structure and function dentification methods

- *MALDI-TOF*
- *Quantitative SDS-PAGE*
- *Quantitative Immunoblot*

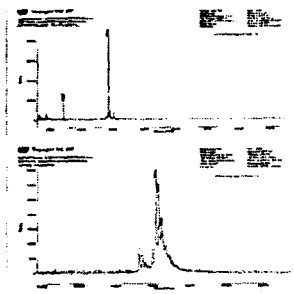
trix-assisted laser desorption/ionization e-of-flight mass spectrometry (MALDI-TOF)



ALDI-TOF analysis of bee nom proteins

ellitin

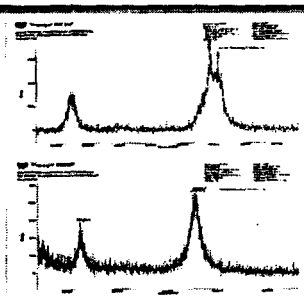
phospholipase



ALDI-TOF analysis of bee nom proteins

aluronidase

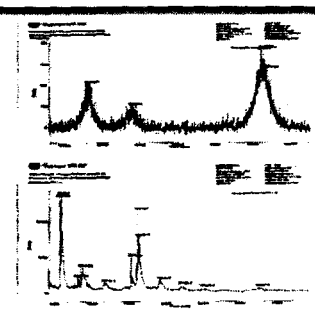
acid phosphatase



ALDI-TOF analysis of bee nom proteins

allergen C

- bee venom



lergen identification techniques dditional questions

- *Can we develop a quantitative profile of natural allergen preparations?*
- *Can we use MALDI-TOF to carefully assess the glycosylation of recombinant allergens?*

munomodulation pitope specific therapy

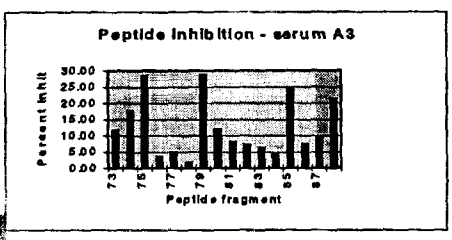
- *Human epitope analysis of Hev b 5*
  - *Site directed mutagenesis*
- *Support for clinical trials of latex immunotherapeutic reagents*

pitope-based immunotherapy

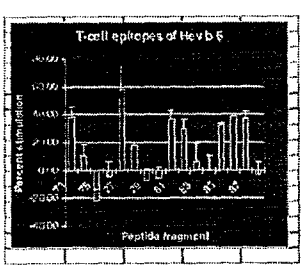
- *Identify and purify antigen*
- *Identify T-cell epitopes of the antigen*
- *Identify B-cell epitopes (IgE-binding sites) of the antigen*
- *Administer immunotherapy with he T-cell epitopes*

r J.E., Paupore E.J. and O'Hehir R.E. (1999) ne B-cell and T-cell epitopes of the allergen b 5 from natural rubber latex *Molecular Immunology* (In Press)

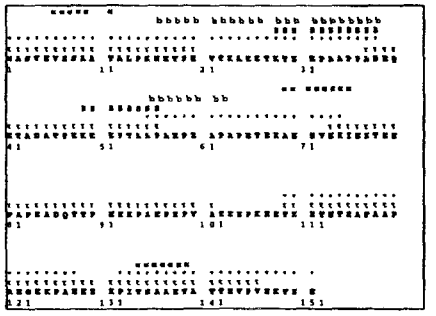
tibody-binding regions of Hev b 5



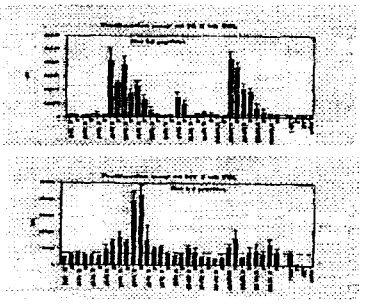
cell epitopes of Hev b 5



Epitope analysis of Hev b 5



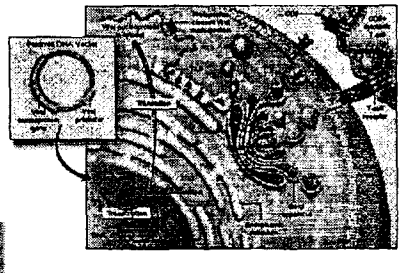
cell epitopes of Hev b 5 in mans (Rolland and O'Hehir)



itopes of Hev b 5 - conclusions

- *Murine B-cell and T-cell epitopes of Hev b 5 have been identified*
- *Preliminary identification of human T-cell epitopes suggests dominance (peptides 37-56, 73-92, 109-128 and 118-137)*
- *Regions for mouse immunotherapy tudy identified*

NA vaccines for allergen munotherapy



ior experience with DNA ccines to reduce IgE responses

- β-galactosidase (Raz et al., *PNAS* 1996; 93:5141)
- Der p 5 (Hsu et al., *Nature Medicine* 1996;2:540)

dvantages of DNA-based IT

- *Th1 response*
- *prolonged expression (>6 months)*
- *multiple antigens can be encoded on a single plasmid*

oblems with DNA-based IT

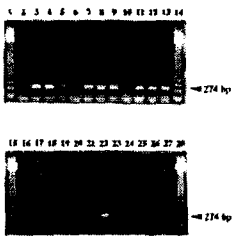
- *unproven safety profile*
  - *mutagenesis*
  - *tissue specificity*
  - *allergen release*
  - *CD8 responses*
- *control of responses in vivo*

eliminary experience with Hev 5 DNA vaccine

- the sense construct is toxic to presensitized mice when injected into the tongue
- no toxicity was noted when the construct was injected intradermally

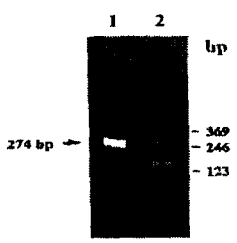
Slater J.E., Paupore E., Zhang Y.T. and Colberg-Poley A.M. (1998) The latex allergen Hev b 5 transcript is widely distributed after subcutaneous injection in BALB/c mice of its DNA vaccine *J Allergy Clin Immunol* **102, 469-475**

v b 5 transcript in tissues of ected mice



striction digest of Hev b 5 RT- R products

SfaNI
'..GCATC(N)$_5$...3'
'..CGTAG(N)$_9$...5'
274 --->155 + 119



NA vaccine therapy - further udies

Construction of DNA vaccines for Hev b 5 using

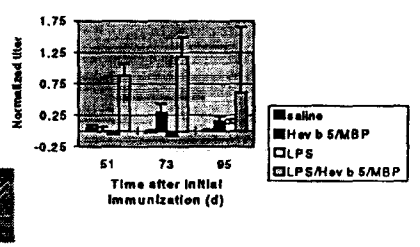
- *specific T-cell epitopes*
- *weak promoters*
- *tissue-specific promoters*

r J.E., Paupore E.J., Elwell M.R. and Truscott
1998) Lipopolysaccharide augments IgG and
esponses of mice to the latex allergen Hev b 5
ergy Clin Immunol 102, 977-983.

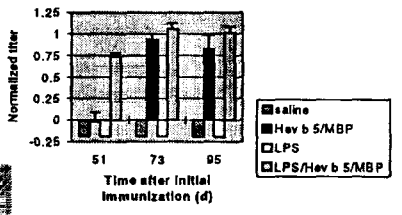
sal immunization protocol

- *BALB/c mice received either **saline, LPS, Hev b 5, or LPS + Hev b 5** (10 μg each)*
- *Anesthetized with methoxyflurane*
- *Received qod doses on **days 1-12** and **days 64-68***

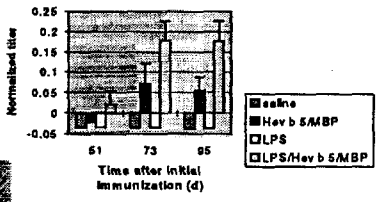
sal immunizations - IgE response



sal immunizations - IgG1 response



sal immunizations - IgG2a response



nunomodulation: lipopolysaccharides onclusions

LPS co-administered with Hev b 5/MBP accentuates

- *anti-Hev b 5 IgE and IgG responses*
- *anti-Hev b 5 and anti-MBP splenocyte proliferation*

nunomodulation: lipopolysaccharides rther questions

- *Does the effect of LPS on antibody production have a functional correlate?*
- *Is the amount of LPS in latex glove powder significant?*
- *Are these effects strain- or antigen-specific?*
- *Is the amount of LPS in allergen xtracts significant?*

B research program summary

- *Allergen structure and function*
  - *Glycosylation*
  - *Enzyme activity*
  - *Identification methods*
- *Immunomodulation*
  - *Epitopes*
  - *DNA vaccines*
  - *LPS*
  - *Cross-sensitization*

boratory of
munobiochemistry

Regulatory proposals

tency limits for standardized ergens - current model

- RID
  - cat
  - short ragweed
- competitive ELISA
  - mites
  - grasses

tency limits for standardized ergens - current model

- Limits are driven by technique
  - skin tests:
    - –wheal: 0.27 to 3.67 (13 X)
    - –erythema: 0.54 to 1.86 (3.4 X)
  - RAST inhibition: 0.46 to 2.12 (4.6X)
  - ELISA inhibition: 0.70 to 1.43 (2X)
- Identical limits for industry and CBER

Failure rates increase as samples fall nearer to limits

tency limits for standardized ergens - equivalence ranges

- *Therapeutic*
  - *up to ten-fold*
- *Diagnostic*
  - *erythema: three- to four-fold*
  - *wheal: up to ten-fold*
- *Safety*
  - *four-fold*

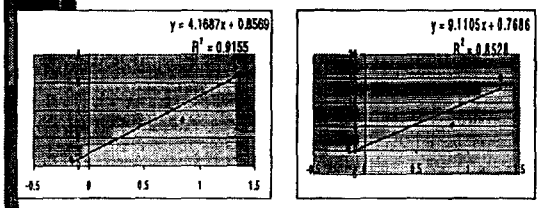
tency limits for standardized allergens nalysis of the safety data: limitations

- *limited numbers of studies*
- *limited numbers of subjects*
- *few with highly allergic subjects*
- *few with standardized allergens*
- *few with consistently defined endpoints*

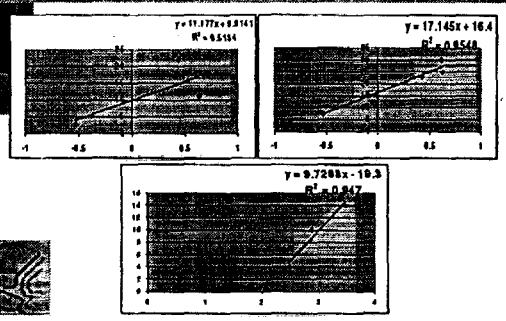
tency limits for standardized allergens nalysis of the safety data: methods

- *identify adverse reaction rates at therapeutic doses*
- *determine increase in adverse reaction rates with log dose increases*
- *separate analysis of "per injection" and "per patient" data*
- *pool data by averaging or weighted averaging; logistic analysis*

tency limits for standardized allergens nalysis of the safety data



tency limits for standardized allergens nalysis of the safety data



tency limits for standardized allergens nalysis of the safety data

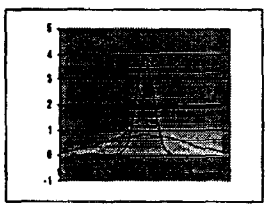
	slope (Δ percent/Δ log dose ± SE)
Averages	
• *per patient:*	*13.4 ± 3.7*
• *per injection:*	*8.2 ± 2.1*
• *all data:*	*10.3 ± 2.1*
■ *Weighted averages*	
• *per patient:*	*13.6 ± 5.5*
• *per injection:*	*5.9 ± 4.3*
• *all data:*	*9.3 ± 3.4*

ency limits for standardized allergens gistic analysis

- *ln (p/(1-p)) = m (ln x) + b*
- *relationship between p and ln x varies*
- *treat data separately*
- *range that yields a 5% increase in reactions (at geometric mean doses)*
  - *Haugaard* 4.6x
  - *Haugaard (maintenance)* 2.4x
  - *Turkeltaub* 5.0x
  - *Turkeltaub (epinephrine)* 1.7x

ow tightly should we regulate ergens?

- *What is the σ of the products that are sent to us? How does it compare to the σ of the assay?*
- *Assuming Gaussian distributions: σ(obs)² = σ(CBER)² + σ(manu)²*

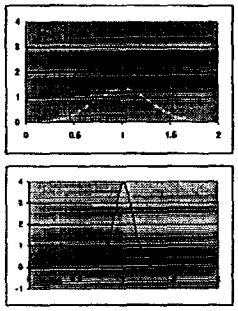


timate of σ of submitted products

- *From 1995-1997, 53/414 or 13% of extracts failed.*
- *σ(obs) = 0.12*
- *σ(CBER) = 0.1375/√3 = 0.08*
- *σ(manu) = √σ(obs)² - σ(CBER)²*
- *σ(manu) = 0.092*

ow tightly should we regulate ergens?

- If the σ of the products that are sent to us is high, we need to insist on equivalence to reference at α
- On the other hand, if the σ of the products that are sent to us is low, we need to test at boundaries to eliminate outliers



tency limits for standardized allergens kelihood of lot differences

For Gaussian distribution

- $r_{mean} = 0.798\sigma$
- $r_{95\%} = 2.77\,\sigma$

- When $\sigma = 0.1$
  - $r_{mean} = 0.798 * 0.1$
  - $= 0.08$ [log]
  - $= 1.2$
  - $r_{95\%} = 2.77 * 0.1$
  - $= 0.28$ [log]
  - $= 1.9$

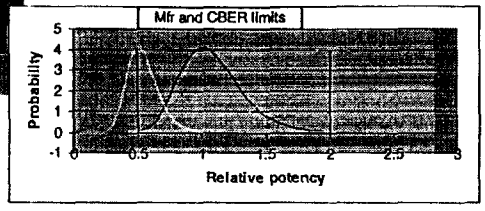
tency limits for standardized ergens - new limit proposal

CBER limits: 0.5 to 2.0

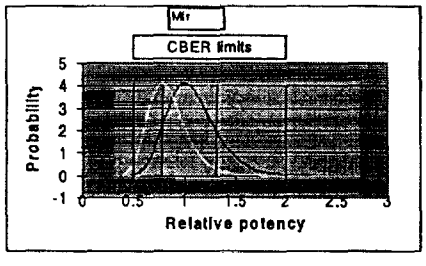
Manufacturer internal limits: unchanged

- for N = 3, 0.7 to 1.43
- for N = 6, 0.78 to 1.29

tribution of samples at the limit rgins - $limits_{CBER} = limits_{manufacturers}$



tribution of samples at the limit rgins - $limits_{CBER} > limits_{manufacturers}$



tency limits for standardized allergens kelihood of product failure

N(manu) = 3	
RP	P(pass)
0.5	0.500
0.6	0.760
0.699	**0.902**
0.7	0.903
0.8	0.965
1	0.993
1.2	0.976
1.3	0.952
1.4	0.916
1.431	**0.902**
1.6	0.806
1.8	0.658
2	0.500

N(manu) = 6	
RP	P(pass)
0.5	0.500
0.6	0.792
0.7	0.934
0.776	**0.975**
0.8	0.982
1	0.998
1.2	0.989
1.288	**0.975**
1.3	0.973
1.4	0.944
1.6	0.841
1.8	0.681
2	0.500

ency limits for standardized allergens - nparison of proposed and current limits

- *Current limits*
  - *0.70 to 1.43 (n=3)*
  - *0.78 to 1.29 (n=6)*
  - *Same for manufacturers and CBER*
  - *technique-driven*
- *Proposed limits*
  - *manufacturers:*
    - *0.70 to 1.43 (n=3)*
    - *0.78 to 1.29 (n=6)*
  - *CBER: 0.5 to 2.0*
  - *study-driven*

otein measurements in allergen tracts - current standard

modified ninhydrin assay

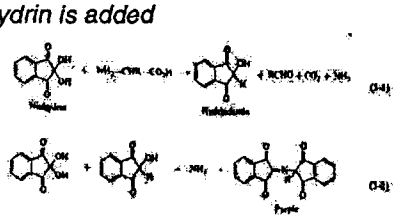
- Richman PG, Cissel DS. A procedure for total protein determination with special application to allergenic extract standardization. *J Biol Stand* 1988; 16:225-238.

- *informational*
- *CBER value must fall within 40% of manufacturer's value*

otein measurements in allergen tracts - current standard

protein hydrolyzed under alkaline conditions, cooled and neutralized

ninhydrin is added



asons to keep a protein ndard

- *purity assessment*
- *alert for the presence of foreign antigens*
- *internal QC*
- *estimate of protein content for other assays*
- *possible effect of protein content on assay*

otein measurements in allergen racts - problems with ninhydrin

- *cumbersome assay*
- *may be overly sensitive*

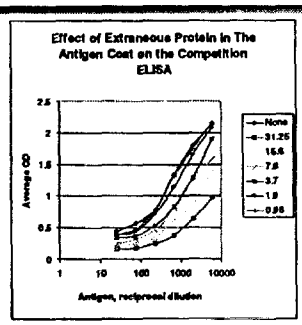
otein measurements in allergen racts - problems with other methods

- *glycerol may affect assay*
- *other chemicals may affect assay*
- *requirement for particular amino acids (tyrosine, tryptophan and cysteine)*

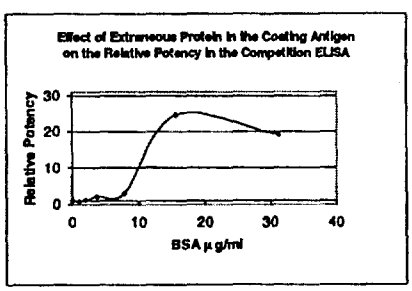
otein interference assay design

- *BSA added, up to 125 µg/mL, in coating step or inhibition step*
- *competitive ELISA*
  - *antigen:* D. pteronyssinus

ecific projects - protein erference with ELISA

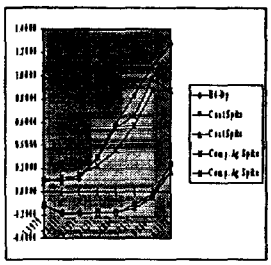


ecific projects - protein erference with ELISA



otein interference assay

- Minimal effect of BSA on competition step
  - protein concentration unlikely to be important lot release criterion
- Substantial effect on coating step, even at lowest concentrations
  - CBER will need to conserve protein concentrations when changing references



asons to keep the protein ndard

- purity assessment
- alert for the presence of foreign antigens
- internal QC
- estimate of protein content for other assays
- possible effect of protein content on assay

otein measurements in allergen racts - suggested revised policy

- continue the requirement for an "informational" protein assay
  - CBER will use these data to ensure consistency of reference standards
- manufacturers may choose any established, validated protein assay
- CBER will no longer routinely assay protein content or reject samples based on protein assay results

otein measurements in allergen racts - revision

- Advantages
  - data will be, within a given manufacturer, internally comparable
  - LIB will not replicate data as part of routine lot release
  - no possibility of lot failure based on the ssay
- Disadvantages
  - protein data will not be comparable among the different manufacturers

otein standard - clarification

- This recommendation applies to standardized mite and grass allergen vaccines only.
- Standardized hymenoptera venoms will continue to be assayed by the ninhydrin assay as currently required.
- The results of protein assays performed on standardized mite and grass allergen vaccines may not be used in product labeling materials.

B objectives 1999-2000

- Regulatory activities
  - continued staff stability/expansion
  - active improvement of support program for standardized allergens
  - support for future standardization efforts (per Advisory Committee recommendations)
- Research activities
  - glycoproteins
  - acid phosphatase
  - MALDI-TOF
  - Hev b 5 epitopes
  - DNA vaccines
  - lipopolysaccharides
  - latex cross sensitization

Jay E. Slater, MD
February 1999

Abbreviations:

αα	amino acid residues
APMA	Allergen Product Manufacturers Association
BALB/c	an inbred strain of albino mice
BSA	bovine serum albumin
Bv	baculovirus
CBER	the Center for Biologics Evaluation and Research, FDA
Der f 1 and 2	allergens from the dust mite *Dermatophagoides farinae*
Der p 1, 2, and 5	allergens from the dust mite *Dermatophagoides pteronyssinus*
E5-Df	reference extract E5 from *Dermatophagoides farinae*
E5-Dp	reference extract E5 from *Dermatophagoides pteronyssinus*
E8-latex	reference extract E8 from *Hevea brasiliensis* latex
Ec	*E. coli*
ELISA	enzyme liked immunosorbent assay
Hev b 1 through 7	allergens from *Hevea brasiliensis* latex
Hya	hyaluronidase
IgG and IgE	immunoglobulins G and E
IT	immunotherapy
LIB	Laboratory of Immunobiochemistry, Division of Allergenic Products and Parasitology, CBER, FDA
LPS	lipopolysaccharide
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MBP	maltose binding protein
n (prefix)	native, or natural

PBS	phosphate buffered saline
RT-PCR	reverse transcriptase polymerase chain reaction
Prs a 1	an allergen from avocado (*Persea americana*)
QC	quality control
r (prefix)	recombinant
r_{mean} and $r_{95\%}$	the ratio of the RPs of two sequential lots of allergen extracts, from a population of extracts whose RPs are represented by a Gaussian distribution around a mean RP of 1. r_{mean} is the average of all r's; and $r_{95\%}$ is the r value below which 95% of sequential lots will fall.
RAST	radioallergosorbent test
RID	radial immunodiffusion
RP	relative potency
σ(obs), σ(CBER), and σ(manu)	standard deviations of the observed allergen extracts, of the CBER assay, and of the manufacturers' submitted products
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Th1	a subset of helper T-cells
TMB	colorimetric substrate for horseradish peroxidase

Revised 10/16/98

Reference Replacement: Track 1

E6-Dp			
	Date	# Days to Complete	Procedure
Start Date	***11/1/98***	7	Select Candidate(s)
	11/08/98	28	Initial CBER Testing
	12/06/98	7	Data Analysis/Select Candidate for Mfr Testing
	12/13/98	14	Hold & Purchase/Call & Send to Mfrs
	12/27/98	84	Mfr testing
	03/21/99	14	Data Analysis
	04/04/99	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**04/18/99**	168	

E4-Or			
	Date	# Days to Complete	Procedure
Start Date	***4/18/99***	7	Select Candidate(s)
	04/25/99	28	Initial CBER Testing
	05/23/99	7	Data Analysis/Select Candidate for Mfr Testing
	05/30/99	14	Hold & Purchase/Call & Send to Mfrs
	06/13/99	84	Mfr testing
	09/05/99	14	Data Analysis
	09/19/99	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**10/03/99**	168	

E4-Sv			
	Date	# Days to Complete	Procedure
Start Date	***10/3/99***	7	Select Candidate(s)
	10/10/99	28	Initial CBER Testing
	11/07/99	7	Data Analysis/Select Candidate for Mfr Testing
	11/14/99	14	Hold & Purchase/Call & Send to Mfrs
	11/28/99	84	Mfr testing
	02/20/00	14	Data Analysis
	03/05/00	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**03/19/00**	168	

E4-Mf			
	Date	# Days to Complete	Procedure
Start Date	***3/19/00***	7	Select Candidate(s)
	03/26/00	28	Initial CBER Testing
	04/23/00	7	Data Analysis/Select Candidate for Mfr Testing
	04/30/00	14	Hold & Purchase/Call & Send to Mfrs
	05/14/00	84	Mfr testing
	08/06/00	14	Data Analysis
	08/20/00	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**09/03/00**	168	

Reference Replacement: Track 1, Page 2

E5-Ber			
	Date	# Days to Complete	Procedure
Start Date	***9/3/00***	7	Select Candidate(s)
	09/10/00	28	Initial CBER Testing
	10/08/00	7	Data Analysis/Select Candidate for Mfr Testing
	10/15/00	14	Hold & Purchase/Call & Send to Mfrs
	10/29/00	84	Mfr testing
	01/21/01	14	Data Analysis
	02/04/01	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**02/18/01**	168	

E7-Df			
	Date	# Days to Complete	Procedure
Start Date	***2/18/01***	7	Select Candidate(s)
	02/25/01	28	Initial CBER Testing
	03/25/01	7	Data Analysis/Select Candidate for Mfr Testing
	04/01/01	14	Hold & Purchase/Call & Send to Mfrs
	04/15/01	84	Mfr testing
	07/08/01	14	Data Analysis
	07/22/01	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**08/05/01**	168	

Reference Replacement: Track 2

C7-Cat			
	Date	# Days to Complete	Procedure
Start Date	***11/1/98***	7	Select Candidate
	11/08/98	14	Initial CBER Testing
	11/22/98	28	Dilute for Std Curve & Test
	12/20/98	7	Data Analysis
	12/27/98	7	Call & Send to Mfrs
	01/03/99	84	Mfr testing
	03/28/99	14	Data Analysis
	04/11/99	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**04/25/99**	175	

E4-Rt			
	Date	# Days to Complete	Procedure
Start Date	***4/25/99***	7	Select Candidate(s)
	05/02/99	28	Initial CBER Testing
	05/30/99	7	Data Analysis/Select Candidate for Mfr Testing
	06/06/99	14	Hold & Purchase/Call & Send to Mfrs
	06/20/99	84	Mfr testing
	09/12/99	14	Data Analysis
	09/26/99	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**10/10/99**	168	

C11-Ras			
	Date	# Days to Complete	Procedure
Start Date	***10/10/99***	7	Select Candidate
	10/17/99	14	Initial CBER Testing
	10/31/99	28	Dilute for Std Curve & Test
	11/28/99	7	Data Analysis
	12/05/99	7	Call & Send to Mfrs
	12/12/99	84	Mfr testing
	03/05/00	14	Data Analysis
	03/19/00	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**04/02/00**	175	

E7-Ti			
	Date	# Days to Complete	Procedure
Start Date	***4/2/00***	7	Select Candidate(s)
	04/09/00	28	Initial CBER Testing
	05/07/00	7	Data Analysis/Select Candidate for Mfr Testing
	05/14/00	14	Hold & Purchase/Call & Send to Mfrs
	05/28/00	84	Mfr testing
	08/20/00	14	Data Analysis
	09/03/00	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**09/17/00**	168	

Reference Replacement: Track 2, Page 2

E12-Rye			
	Date	# Days to Complete	Procedure
Start Date	***9/17/00***	7	Select Candidate(s)
	09/24/00	28	Initial CBER Testing
	10/22/00	7	Data Analysis/Select Candidate for Mfr Testing
	10/29/00	14	Hold & Purchase/Call & Send to Mfrs
	11/12/00	84	Mfr testing
	02/04/01	14	Data Analysis
	02/18/01	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**03/04/01**	168	

E5-Jkb			
	Date	# Days to Complete	Procedure
Start Date	***3/4/01***	7	Select Candidate(s)
	03/11/01	28	Initial CBER Testing
	04/08/01	7	Data Analysis/Select Candidate for Mfr Testing
	04/15/01	14	Hold & Purchase/Call & Send to Mfrs
	04/29/01	84	Mfr testing
	07/22/01	14	Data Analysis
	08/05/01	14	Final Decision; Memo & Shipment to Mfr
Completion Date	**08/19/01**	168	

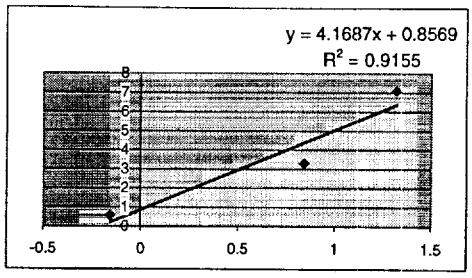
Release limits data - log 10 analyses

Therapeutic range data only

Haugaard data
total data
semilog

0.7	-0.1549	0.56
7	0.845098	3.3
21	1.322219	7.1

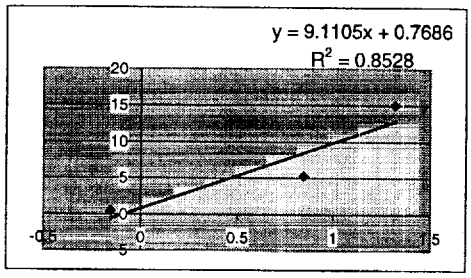
m	b	4.168735	0.856925
Sem	SEb	1.266292	1.152824
r2	SEy	0.915525	1.349959
F	df	10.8378	1
ssreg	sesresid	19.75068	1.822389



Haugaard data
maintenance only
semilog

0.7	-0.1549	0.4
7	0.845098	5.24
21	1.322219	15

m	b	9.110539	0.768604
Sem	SEb	3.784991	3.445832
r2	SEy	0.852805	4.035075
F	df	5.793732	1
ssreg	sesresid	94.33257	16.28183

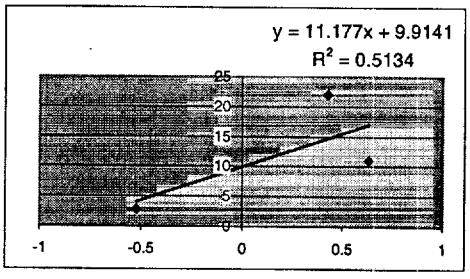


Turkeltaub data
semilog

0.003	-2.52288	2.3
0.3	-0.52288	2.8
2.7	0.431364	22
4.3	0.633468	11

Drop the 0.003 point

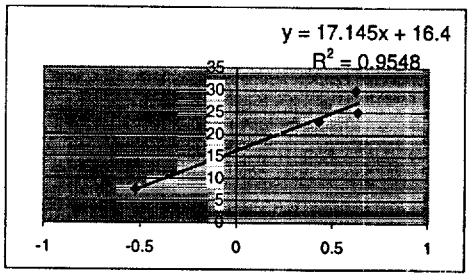
m	b	11.17747	9.914111
Sem	SEb	10.88098	5.828387
r2	SEy	0.513438	9.503621
F	df	1.055238	1
ssreg	sesresid	95.30786	90.31881



Turkeltaub epi data
semilog

0.3	-0.52288	7.5
0.82	-0.08619	15
2.7	0.431364	23
4.2	0.623249	30
4.3	0.633468	25

m	b	17.14537	16.39997
Sem	SEb	2.153875	1.079789
r2	SEy	0.954796	2.179326
F	df	63.36544	3
ssreg	sesresid	300.9516	14.24838

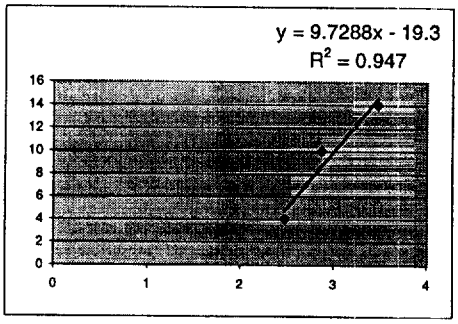


Lopez
semilog cumulative

30	1.477121	2
75	1.875061	2
300	2.477121	4
750	2.875061	10
3000	3.477121	14

top three points only

m	b	9.728803	-19.2995
Sem	SEb	2.301228	6.838491
r2	SEy	0.947015	1.638474
F	df	17.87309	1
ssreg	sesresid	47.98207	2.684598



Equal weight averages

Per injection summary

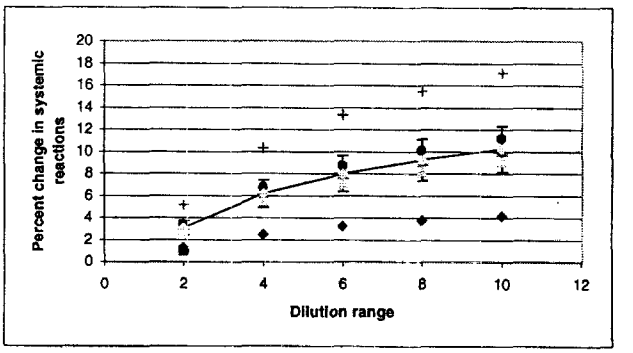
	slope	SE
Haugaard all data	4.17	1.27
Haugaard(all data	9.11	3.78
Turkeltaub worst case	11.18	10.88

Per patient summary

	slope	SE
Turkeltaub epi data	17.15	2.15
Lopez worst case	9.73	2.30

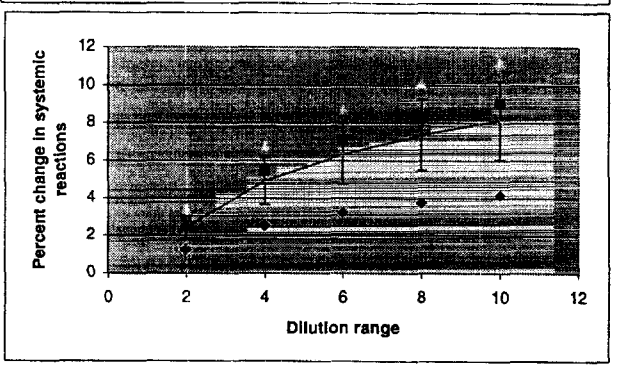
All data

	2	4	6	8	10
	1.254914	2.509828	3.243906	3.764743	4.168735
	2.742546	5.485091	7.089377	8.227637	9.110539
	3.364753	6.729507	8.697761	10.09426	11.17747
	5.16127	10.32254	13.34169	15.48381	17.14537
	2.928662	5.857323	7.57048	8.785985	9.728803
Average	3.090429	**6.180858**	7.988643	9.271287	**10.26618**
SD	1.403562	**2.807124**	3.628155	4.210685	**4.662531**
SE	0.627692	**1.255384**	1.62256	1.883076	**2.085147**



per injection only

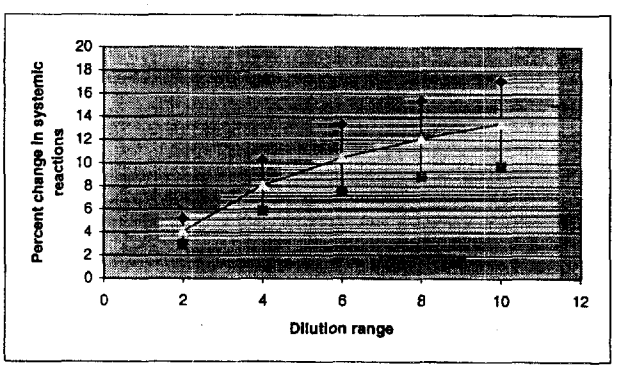
	2	4	6	8	10
	1.254914	2.509828	3.243906	3.764743	4.168735
	2.742546	5.485091	7.089377	8.227637	9.110539
	3.364753	6.729507	8.697761	10.09426	11.17747
Average	2.454071	**4.908142**	6.343682	7.362213	**8.152248**
SD	1.084098	**2.168196**	2.802353	3.252294	**3.601296**
SE	0.625904	**1.251809**	1.617939	1.877713	**2.079209**



per patient only

M

	2	4	6	8	10
	5.16127	10.32254	13.34169	15.48381	17.14537
	2.928662	5.857323	7.57048	8.785985	9.728803
Average	4.044966	**8.089932**	10.45609	12.1349	**13.43709**
SD	1.578693	**3.157385**	4.080861	4.736078	**5.244303**
SE	1.116304	**2.232609**	2.885605	3.348913	**3.708283**



Weighted averages

Per injection summary

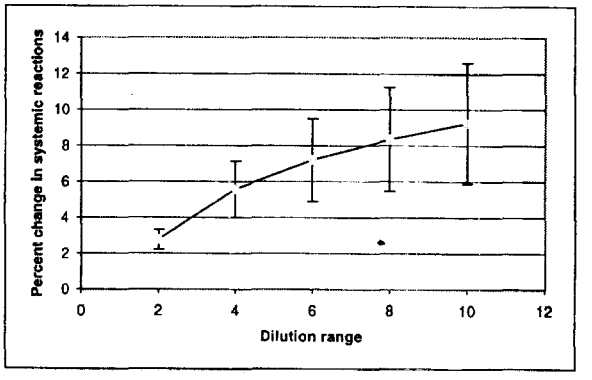
	slope	SE
Haugaard all data	4.17	1.27
Haugaard(all data	9.11	3.78
Turkeltaub worst case	11.18	10.88

Per patient summary

	slope	SE
Turkeltaub epi data	17.15	2.15
Lopez worst case	9.73	2.30

All data

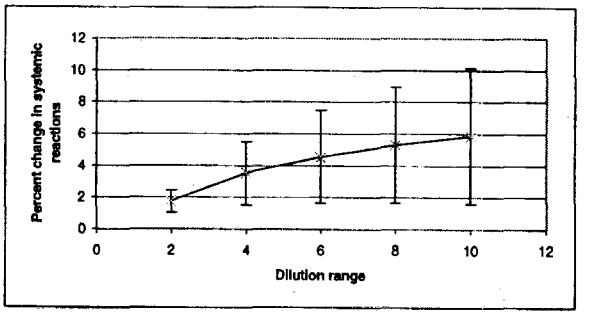
M	2	4	6	8	10
	1.254914	2.509828	3.243906	3.764743	4.168735
	2.742546	5.485091	7.089377	8.227637	9.110539
	3.364753	6.729507	8.697761	10.09426	11.17747
	5.16127	10.32254	13.34169	15.48381	17.14537
	2.928662	5.857323	7.57048	8.785985	9.728803
Waverage	2.784719	**5.569439**	7.198395	8.354158	**9.250637**
WSD	1.444579	**2.889158**	3.734183	4.333737	**4.798788**
WSE	0.554291	**1.567771**	2.303663	2.880179	**3.356007**
Sem	2	4	6	8	10
	0.381192	0.762384	0.985366	1.143575	1.266292
	1.139396	2.278792	2.945296	3.418188	3.784991
	3.275503	6.551006	8.467052	9.826508	10.88098
	0.648381	1.296762	1.676041	1.945143	2.153875
	0.692739	1.385477	1.790703	2.078216	2.301228



per injection only

M

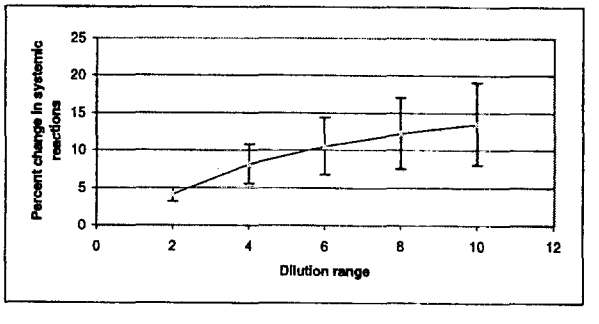
	2	4	6	8	10
	1.254914	2.509828	3.243906	3.764743	4.168735
	2.742546	5.485091	7.089377	8.227637	9.110539
	3.364753	6.729507	8.697761	10.09426	11.17747
Waverage	1.767158	**3.534317**	4.568038	5.301475	**5.870373**
WSD	1.37224	**2.744479**	3.547188	4.116719	**4.558482**
WSE	0.703361	**1.989405**	2.923207	3.65477	**4.258567**
Sem	2	4	6	8	10
	0.381192	0.762384	0.985366	1.143575	1.266292
	1.139396	2.278792	2.945296	3.418188	3.784991
	3.275503	6.551006	8.467052	9.826508	10.88098



per patient only

M

	2	4	6	8	10
	5.16127	10.32254	13.34169	15.48381	17.14537
	2.928662	5.857323	7.57048	8.785985	9.728803
Waverage	4.081888	**8.163775**	10.55153	12.24566	**13.55974**
WSD	1.579556	**3.159112**	4.083093	4.738668	**5.247171**
WSE	0.914116	**2.585509**	3.799115	4.749884	**5.534603**
Sem	2	4	6	8	10
	0.648381	1.296762	1.676041	1.945143	2.153875
	0.692739	1.385477	1.790703	2.078216	2.301228

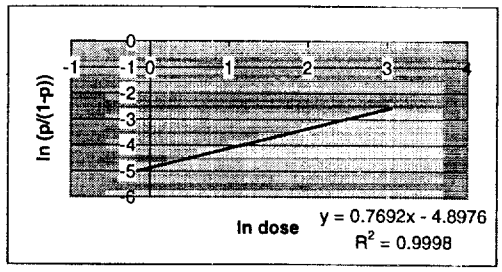


Logistic analysis

Haugaard data
total data

	dose	ln dose	p (%)	p	ln(p/(1-p))
	0.7	-0.356675	0.56	0.0056	-5.179373
	7	1.94591	3.3	0.033	-3.377691
	21	3.044522	7.1	0.071	-2.571429

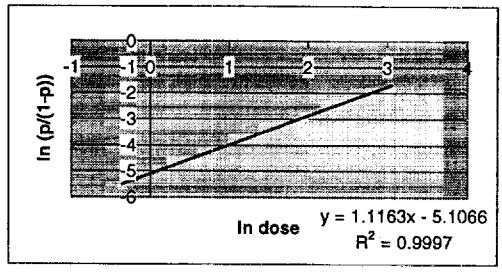
m	b	0.769178	-4.897559
Sem	SEb	0.011772	0.024677
r2	SEy	0.999766	0.028897
F	df	4269.166	1
ssreg	sesresid	3.564995	0.000835



Haugaard data
maintenance only

	dose	ln dose	p (%)	p	ln(p/(1-p))
	0.7	-0.356675	0.4	0.004	-5.517453
	7	1.94591	5.24	0.0524	-2.895026
	21	3.044522	15	0.15	-1.734601

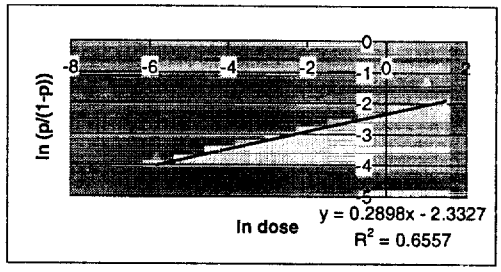
m	b	1.116305	-5.10659
Sem	SEb	0.020031	0.041989
r2	SEy	0.999678	0.049169
F	df	3105.859	1
ssreg	sesresid	7.508808	0.002418



Turkeltaub

	dose	ln dose	p (%)	p	ln(p/(1-p))
	0.003	-5.809143	2.3	0.023	-3.748992
	0.3	-1.203973	2.8	0.028	-3.547151
	2.7	0.993252	22	0.22	-1.265666
	4.3	1.458615	11	0.11	-2.090741

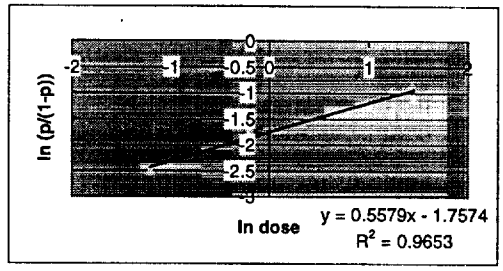
m	b	0.289809	-2.332665
Sem	SEb	0.148506	0.459589
r2	SEy	0.655667	0.854506
F	df	3.80833	2
ssreg	sesresid	2.780765	1.460359



Turkeltaub epi data

	dose	ln dose	p (%)	p	ln(p/(1-p))
	0.3	-1.203973	7.5	0.075	-2.512306
	0.82	-0.198451	15	0.15	-1.734601
	2.7	0.993252	23	0.23	-1.208311
	4.2	1.435085	30	0.3	-0.847298
	4.3	1.458615	25	0.25	-1.098612

m	b	0.557871	-1.757435
Sem	SEb	0.061092	0.070521
r2	SEy	0.965272	0.142332
F	df	83.38634	3
ssreg	sesresid	1.689284	0.060776



ln (p/(1-p)) = m(ln x) + b

Study	geo mean	m	b	ln p/(1-p)	p/(1-p)	p	p + .05	ln p/(1-p)	new dose	factor
Haugaard	4.69	0.77	-4.90	-3.71	0.02	0.02	0.07	-2.53	21.77	**4.64**
Haugaard (maintenance)	4.69	1.12	-5.11	-3.38	0.03	0.03	0.08	-2.40	11.26	**2.40**
Turkeltaub	1.52	0.29	-2.33	-2.21	0.11	0.10	0.15	-1.75	7.59	**5.01**
Turkeltaub (epi)	1.64	0.56	-1.76	-1.48	0.23	0.19	0.24	-1.18	2.82	**1.72**

DEPARTMENT OF HEALTH & HUMAN SERVICES
FDA/CBER/OVRR/DAPP

Memorandum

Date DRAFT

To

From Jay E. Slater, MD, Chief, Laboratory of Immunobiochemistry

Through

Subject Elimination of the requirement for the ninhydrin total protein assay for standardized mite and grass allergen vaccines

The determination of the protein content of allergen extracts has been used as a lot release criterion for standardized allergen vaccines. With advances in allergen standardization and identification, the protein content of an individual vaccine may be of questionable relevance to the safety and efficacy of the product. The purpose of this memorandum is to discuss the advantages and disadvantages of changing the requirement that manufacturers determine the protein content of standardized mite and grass allergen vaccines, and to recommend a specific diminution of the regulatory requirements for this assay.

Reasons for a protein lot release requirement

The most important reason for determining total protein is as a measure of product consistency from lot to lot. Large variations in protein content may signal manufacturing deficiencies that warrant attention. Furthermore, a decrease in the potency/unit protein of an allergen extract may be a sensitive indicator of allergen degradation or contamination.

Another reason to monitor the protein content is the need to estimate the amount of material needed for other laboratory assays of possible regulatory interest. These include analysis by gel electrophoresis, immunoblot, radial immunodiffusion, isoelectric focussing, crossed radial immunoelectrophoresis, HPLC and MALDI-TOF.

Finally, contaminating proteins may interfere with other assays. Primarily, these may affect protein-protein interactions, especially the solid-phase coating step in ELISA-based assays. In addition, other reactions depending on antigen-antibody interactions may be susceptible to unanticipated effects contributed by extraneous proteins.

The choice of a standard protein assay for allegen extracts

Unfortunately, each of the common protein assays has limitations that are of special concern when evaluating allergen vaccines. Glycerol, a common component of allergen preparations, interferes with Lowry-based assays (including the BCA assay), and may affect the Coomassie blue-based assays (e.g. Bradford) as well. In addition, these assays are all dependent upon the presence of particular amino acid residues for color development, which may not be present in comparable amounts in all allergens. The latex allergen Hev b 5, for instance, is devoid of tyrosine, tryptophan and cysteine[1], and may be undetectable using these techniques[2].

In consideration of these concerns, CBER developed and adopted a modification of the more cumbersome ninhydrin technique of protein determination[3]. In this assay, the protein is hydrolyzed under alkaline conditions. The mixture is cooled and neutralized, and ninhydrin is added. Ninhydrin elicits the oxidative deamination of the α-amino group, and it is the reduced form of ninhydrin that absorbs light near 570 nm. Unlike other methods, the reaction of ninhydrin with amino acids is largely independent of the side chain; thus, in principle, the ninhydrin assay can closely approximate the results of an amino acid analysis[4].

It is notable that release limits were not established for the protein content of standardized allergen vaccines. Rather, the results of the ninhydrin assay have been required for information only. When the results have been checked as part of the lot release program of LIB, the requirement has been that the results of the CBER assay be within 40% of the manufacturer's result.

Problems associated with the ninhydrin assay

Compared with other available protein assays, the ninhydrin assay is lengthy and difficult. Toxic, caustic reagents are used at high temperatures. Hydrolysis is achieved by the addition of 10 N NaOH and incubation in a 150°C oven. Continued incubation of open tubes at 110°C is necessary to eliminate free ammonia and decrease background signal in the assay. The mixture is neutralized with 10 N acetic acid before the ninhydrin reagent is added[3].

Furthermore, the sensitivity of the ninhydrin assay may be a limitation. While the standard protein assays are unlikely to detect small peptides and amino acids, the ninhydrin assay will do so. These small peptide sequences may be of less concern than larger proteins that are more likely to be allergenic. Furthermore, by increasing the detection of small peptides and amino acids, the ninhydrin assay may be less likely than other protein assays to detect shifts in the concentration of proteins of greater immunologic significance[5].

Is the ninhydrin assay necessary to measure extraneous protein?

Theoretically, it is possible that an allergen submitted for lot release will be contaminated with other allergens or extraneous proteins that will be detected only by an increase in the total protein content. However, it is likely that these extraneous proteins will be detected in the identity testing. In addition, following the lot-to-lot total protein content of a product over time can be accomplished as well using one of the standard protein assays. Although glycerol may interfere with the assays, the glycerol content should be relatively stable from lot to lot.

Are total protein assays necessary to establish the amount of allergen to be used for other assays?

Knowing the amount of protein in a sample can save time and expense in running certain assays. Examples include HPLC, IEF and immunoblots, where the reagent, labor and equipment time expenditures are high. However, for ELISA assays, costs are relatively low, multiple dilutions are performed as a matter of course, and the initial concentration is less important. Furthermore, even for those assays for which the initial protein concentration would be useful information, alternative protein assays, when properly validated, can provide adequate information.

Are protein assays necessary to prevent interference with other assays?

We examined the possibility that protein levels may affect the results of the competitive ELISA. We found added protein significantly inhibited the binding of allergen to microtiter wells, but had no measurable effect on the competition step. This suggests that the protein content of an allergen vaccine submitted for lot release will not affect the results of lot release testing. However, the protein content of the candidate allergen vaccine must be considered when CBER selects a reference extract (which is, in all cases, the allergen bound to the wells in the initial step). Once again, alternative protein assays, when properly validated, can be used to monitor the protein content for this purpose.

Thus the ninhydrin assay currently required by CBER for the approval of standardized allergen vaccines is difficult to use, and the assay sensitivity may be of limited utility or relevance. It is probably no longer necessary as a quality control measure, and other protein assays are probably sufficient indicators of extraneous protein content. Initial estimates of protein content are not needed for the radial immunodiffusion or competitive ELISA assays, and the estimates provided by the standard protein assays would be sufficiently accurate for the establishment of initial conditions for tests such as HPLC and IEF. Finally, the protein content of an extract

submitted for lot release does not appear to affect the results of the competitive ELISA assay used by CBER.

Current options (summarized in Table)

1. **No change.** This is the most conservative approach. The main advantage is that CBER will continue to require that each manufacturer utilize the same assay method, and that information on the specific activity (relative potency/unit protein) of allergen vaccines from different manufacturers will be directly comparable. Another advantage is that CBER biologists will need to master only one, albeit difficult, protein assay. CBER has extensive experience with this assay, and has collected large amounts of data from the manufacturers since the initiation of the standardization program. These data could be utilized to initiate future studies of the potency of allergen vaccines. The disadvantage of this approach is that CBER will continue to require the manufacturers to perform a difficult assay using hazardous reagents to collect data that are, at best, of uncertain value. We have no example yet of a production or manufacturing defect that has been uncovered as a result of the data obtained using the protein assay.
2. **Require the ninhydrin assay, but eliminate limits on the protein content.** At present, the only limits that are in force reflect on the accuracy of the assay and of the laboratory personnel who perform the assay, in the manufacturers' laboratories and at CBER. The advantage of this approach is that we will continue to collect comparable, reliable data, which can be used for quality control and assay dosing purposes. However, we eliminate the possibility of lot failure based on the assay, and eliminate the need for LIB to perform the assay as part of the lot release process.
3. **Require a protein assay, permit the choice of any standard protein assay, but set limits on the protein content.** Under this option, we expect that, once an assay is chosen, it will be changed only with adequate justification. The main advantage of this approach is that we will continue to check on the protein content of the vaccines and the accuracy of the manufacturers' laboratory determinations. We will also continue to collect data on allergen vaccines that are, within a given manufacturer, internally comparable. In addition, these data will be helpful in determining initial amounts of allergen to use in various subsequent assays. There is at least a theoretical possibility that the standard protein assays measure larger, more significant allergenic proteins, while the ninhydrin assay measures all proteins and polypeptides. The major disadvantages are that LIB will have to run each of several different protein assays on a routine basis, and the acceptable variations will have to be determined individually for each assay. The protein data will not be comparable among the different manufacturers. Lot release failure on the basis of the protein assay remains a possibility in this option, and it is not clear that such failure has any justifiable basis.
4. **Require any protein assay, but eliminate limits on the protein content.** The advantage of this approach is that we continue to collect data on allergen vaccines that are, within a given manufacturer, internally comparable. These data will be helpful in determining initial amounts of allergen to use in various assays. LIB personnel will only need to replicate the manufacturer's data as part of the investigation of a specific problem, but not as part of routine lot release. Once again, we eliminate the possibility of lot failure based on the assay. However, the protein data will not be comparable among the different manufacturers.
5. **Eliminate protein assay requirement.** The advantage of this approach is that it relieves the industry and LIB of all regulatory burdens associated with the protein content of allergen vaccines. There are several disadvantages. The major disadvantage is that CBER will no longer have any information on the protein content of standardized allergen vaccines, unless the manufacturers voluntarily provide this information. Of lesser importance is that LIB workers will no longer have an initial estimate from the manufacturer of protein content for assays such as IEF, HPLC and MALDI-TOF, and for the selection of appropriately consistent reference standards.

Recommendation

Overall, there appears to be little justification for continuing to require the use of the ninhydrin assay. However, manufacturers should continue to perform protein assays on each lot of material, and CBER should require this information as part of its lot release program. The choice of a protein assay will be left to the manufacturer, which must provide proficiency and validation data on the particular assay, and an SOP that can be followed by CBER personnel should replication be required. Since one of the reasons to require protein data is for ongoing monitoring of particular allergen vaccines, *manufacturers will be expected to use one protein assay consistently* unless compelling reasons are presented to CBER.

Likewise, the advantages of continuing to require that CBER personnel replicate the manufacturer's data within 40% limits are uncertain. This does not represent a true limit on protein content, but rather a test of the accuracy of the manufacturer's assay technique. Appropriate proficiency and validation should be adequate for this purpose.

At this time, there are no data to support the establishment of a true limit on the protein content of allergen vaccines.

The major substantive disadvantage of this option (#4, above) is that the protein data will not be comparable among the different manufacturers. We have no evidence that such data have been used in the past. Furthermore, if a particular issue should arise for which these data are needed, CBER personnel can determine the protein content of allergen vaccines from different manufacturers by performing one of the protein assays (including, at the Lab Chief's discretion, the ninhydrin assay) on lot release samples.

This recommendation applies to standardized mite and grass allergen vaccines only. Standardized hymenoptera venoms will continue to be assayed by the ninhydrin assay as currently required. Furthermore, the results of protein assays performed on standardized mite and grass allergen vaccines may not be used in product labeling materials.

Reference List

1. Slater JE, Vedvick T, Arthur-Smith A, Trybul DE, Kekwick RGO. Identification, cloning and sequence of a major allergen (Hev b 5) from natural rubber latex (Hevea brasiliensis). J.Biol.Chem. 1996; 271:25394-25399.

2. Akasawa A, Hsieh LS, Martin BM, Liu T, Lin Y. A novel acidic allergen, Hev b 5, in latex : purification, cloning and characterization. J.Biol.Chem. 1996; 271:25389-25393.

3. Richman PG, Cissel DS. A procedure for total protein determination with special application to allergenic extract standardization. J Biol Stand 1988; 16:225-238.

4. Mahler HR, Cordes EH. Biological chemistry. 2 ed. New York: Harper & Row, 1971.

5. Stevens E. Principles and practice in the monograph on allergenic extracts of the European Pharmacopoeia. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M 1997; 120-122.

Approach	Lot-to-lot consistency	Industry-wide data	Initial quantities for assays	Control for possible interference with other assays	Assay is easy to perform		Measures mostly proteins >10 kDa
					Mfr	LIB	
1. Status quo	Yes	Yes	Yes	Yes	No	No	No
2. Ninhydrin/no limits	Yes	Yes	Yes	Yes	No	N/A	No
3. Any assay/limits	Yes	No	Yes	Yes	Yes	Yes*	Yes
4. Any assay/no limits	Yes	No	Yes	Yes	Yes	N/A	Yes
5. No requirement	No	No	No	No	N/A	N/A	No

* However, LIB staff will have to perform multiple protein assays, and variance limits will have to be set for each assay.