# Developing an ELISA (Enzyme-linked Immunosorbent Assay) to pig small intestinal mucin

**Natalie Nichols**

## Introduction

Although the portal-drained viscera (PDV, metabolism predominantly intestinal) represents only about 5% of body mass, the tissues account for around 25-30% of whole body protein turnover (Stoll 1999). Indeed, the extraction of dietary amino acids by the PDV has been estimated at 20-70% of dietary essential amino acids on first pass (Stoll 1998). Given the significant demand of the gut for amino acids, it is obvious that gut maintenance and growth constitute a significant proportion of whole body amino acid requirements. It follows that in certain situations, which increase the metabolic activity of the gut (ie increased growth, pathogen exposure, dietary anit-nutritional factors), this proportion will increase.

A major factor in the maintenance of intestine is luminal mucus gel. The mucus coat separates mucosal cells from the exterior components and provides protection for the intestine. Mucus, therefore, plays an important role for the intestinal surface integrity in health. Dysfunction of mucus section may be involved in several pathologies of the intestine. Mucins are the predominant component of this mucus layer. They are high molecular weight glycoproteins with oligosaccharides attached by O-glycosidic bonds to a protein core that is made up of ~40% threonine, proline and sulfur amino acids. In times of nutritional or pathogenic challenges, the gut produces greater amounts of mucin as a defense mechanism. However, it is unknown what effect these challenges have on amino acid requirements of the small intestine or the animal as a whole.

The present study will focus on how various pathogenic and nutritional factors, such as cholera toxin, fiber, casein peptides, etc., act as secretagogues of mucin and what role this plays on amino acid requirements of the piglet. Before this procedure takes place, we will aim to produce an ELISA (enzyme-linked immunosorbent assay) to purified mucin in order to quantify the mucin secretion produced by the secretagogues mentioned above.

In this paper, we will use the data obtained from the purification steps of developing an ELISA. We will use the orcinol assay in this purification step. In this assay, hexose sugar is measured. This assay will allow me to observe the presence of mucin. As well, mucin, when put through a cesium chloride gradient, settles at a certain density (~1.4 g/ml). When the cesium chloride gradient is divided into fractions, each fraction can be then assayed by the orcinol assay to measure where the mucin settled out in the gradient. With this in mind, my statistical question will be to determine if there is consistency between Elisa 3 and 4 in the location or density (which we describe as depth) of mucin.

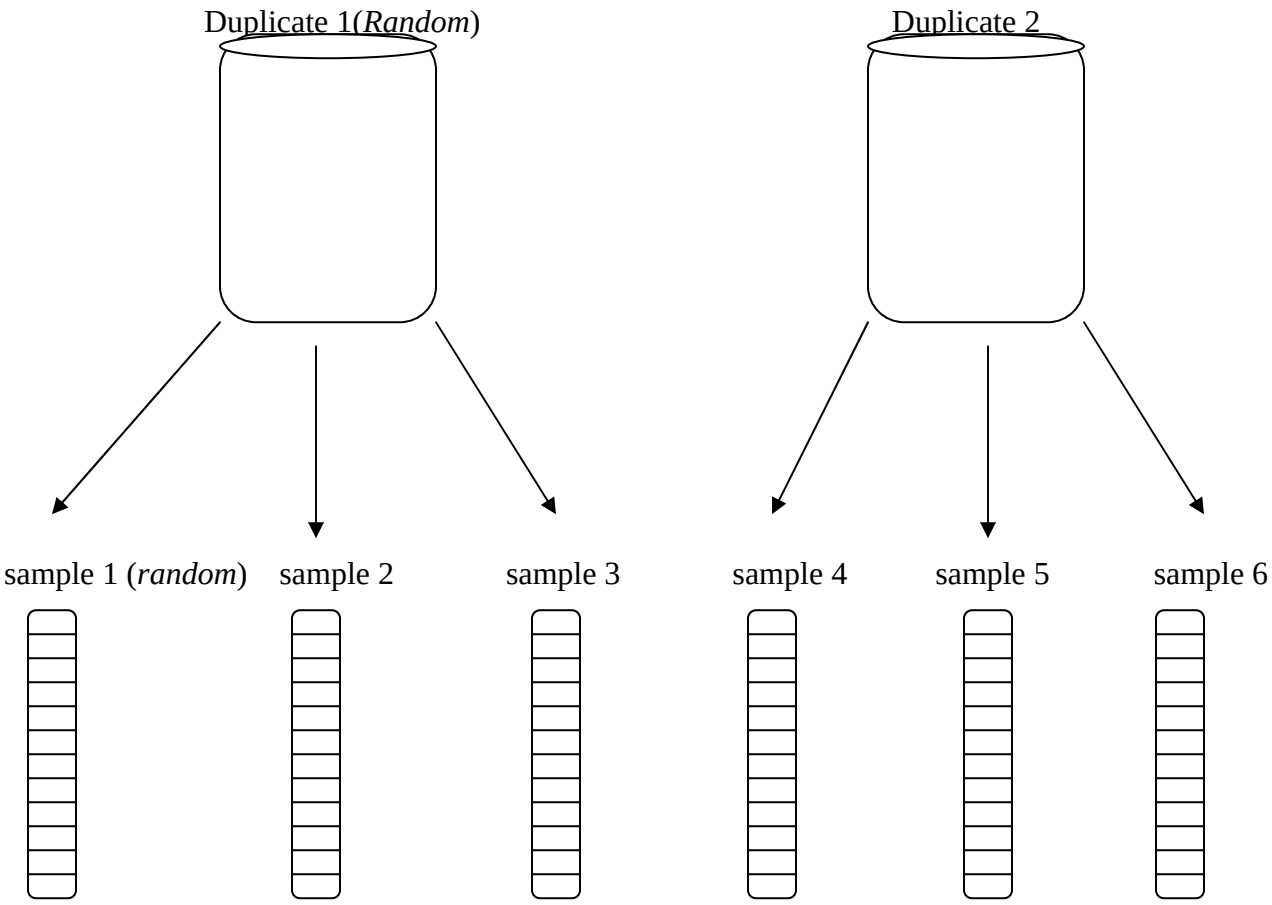
## Methods

**Purification of pig intestinal glycoprotein**

Pig intestinal glycoprotein was purified by a modification of the method of Claustre et al., 2002. Briefly, mucus was collected by gently scraping the small intestinal mucosa from a ~12 kg pig. 8 g of mucus scrapings were solubilized in 80 mls of 50 mM Tris buffer (pH 7.5) containing 6 M guanidinium hydrochloride and 100 mM DTT by stirring in the dark at 4°C for 24 hours. Iodoacetamide (250 mM) was added (to carboxymethylate the sulfhydryl groups) and stirred for an additional 24 hours at 4°C in the dark. The solution was then centrifuged (30 min, 17,000 rpm, 4°C) to remove any insoluble material. Mucins were then purified by equilibrium centrifugation on a cesium chloride density gradient at a density of 1.5 g/ml ( ultracentrifuge, SW 28 rotor, 23, 500 rpm, 70 hr, 12°C). From the gradient, 12 equal density fractions were removed and put through dialysis against $dH_2O$ for 24 hrs at 4°C. The fractions were then assayed for hexose by the Orcinol assay and protein by the Bradford assay.

The first two times this procedure was performed (Elisa 1 and 2) an improper scraping technique was performed and therefore the samples could not be used. The third time, the scraping technique was performed correctly, however, other techniques had to be developed and during this time, mold started to grow in the samples which forced an Elisa 4 to be performed. The data that will be analyzed will be from Elisa 3 and 4. The following is a schematic of the division of my samples due to lack of equipment availability.

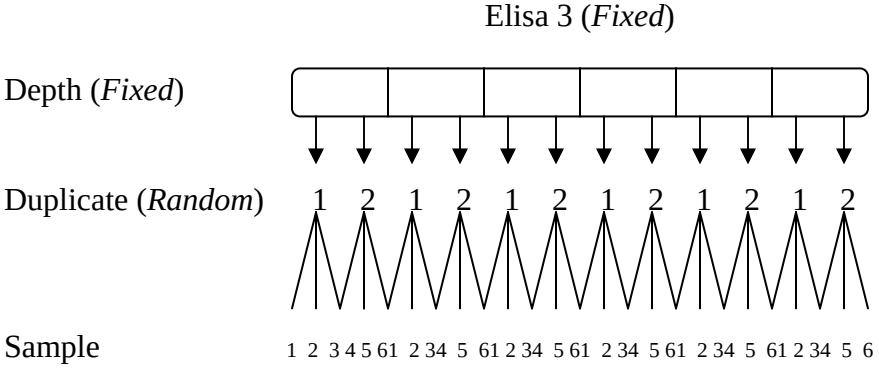
Elisa 3 (exactly the same for Elisa 4)-*Fixed*



*Fixed* - Each sample is divided into 12 fractions which we divided into 6 depths to analyze.

## Analysis

Originally, my statistical question was "how does the quanitity of mucin differ between Elisa 3 and 4?" and if this was statistically significant " how does the quanitity of mucin differ between duplicates within Elisa 3 and within Elisa 4?" A four-way anova was going to be performed using the model of Abs = Elisa + Duplicate + Depth + Sample + Elisa*Duplicate*Depth*Sample. (Absorbance is measured on the mucin samples with a spectrophotometer at a wavelength of 540 nm. Absorbance has no unit value). However, an error term was displayed stating, " * ERROR * Model is non-hierarchical at I. " It was determined that the Minitab program would not allow this analysis to take place since there was a random variable being tested relative to a fixed variable at a lower level. If the previous diagram is observed, it can be seen that the sample variable is random and the depth is a fixed variable. So the data ( and in turn the model) was re-arranged to the following schematic to eliminate this problem.




The model that was used for the design was:

Abs. = Elisa + depth + Elisa*depth + duplicate (Elisa) + sample (duplicate)

This model takes into account the fact that the random variables cannot be tested relative to fixed variables at a lower level. As well, it applies the concept of nesting when analyzing the random factor of duplicate and sample

The results for this analysis were as follows:

```
Factor                   Type    Levels  Values
elisa                    fixed        2  3, 4
depth                    fixed        6  1, 2, 3, 4, 5, 6
duplicate(elisa)         random       4  1, 2, 1, 2
sample(elisa duplicate)  random      12  1, 2, 3, 4, 5, 6, 1, 2, 3, 4, 5, 6

Source                    DF    Seq SS    Adj SS    Adj MS      F      P
elisa                      1  0.174167  0.174167  0.174167   4.25  0.175
depth                      5  0.010794  0.010794  0.002159   1.38  0.236
elisa*depth                5  0.035785  0.035785  0.007157   4.58  0.001
duplicate(elisa)           2  0.082019  0.082019  0.041009  10.10  0.006
sample(elisa duplicate)    8  0.032487  0.032487  0.004061   2.60  0.012
Error                    122  0.190579  0.190579  0.001562
Total                    143  0.525831
```

When the data was analyzed to see if the computations of the F ratio had been properly executed, it was discovered that the F ratio was computed properly for the duplicate(elisa) and sample (elisa duplicate):

$F = \frac{0.004061}{0.001562} = 2.60$ and $F = \frac{0.041009}{0.004061} = 10.10$

However, the remaining F ratios had not been properly computed. They were instead computed by using the formula of $\frac{MS}{MS_{error}}$ but I needed the F ratios to be computed over the MS of the duplicate(elisa) term. So I had to recompute the F-ratios as follows:

Elisa

$F \text{ ratio} = \frac{0.174167}{0.041009} = 4.25$

Depth

$F \text{ ratio} = \frac{0.002159}{0.041009} = 0.0527$

Elisa*Depth

$F \text{ ratio} = \frac{0.007157}{0.041009} = 0.175$

From these F-ratios I can now use the cdf command to calculate the p-values that are of importance to me. The results were as follows:

Elisa

```
F distribution with 1 DF in numerator and 122 DF in denominator
```

```
   x  P( X <= x )
4.25     0.958626
```

Depth

```
F distribution with 5 DF in numerator and 122 DF in denominator

     x  P( X <= x )
0.0527    0.0017729
```

Elisa*depth

```
F distribution with 5 DF in numerator and 122 DF in denominator

    x  P( X <= x )
0.175    0.0285272
```

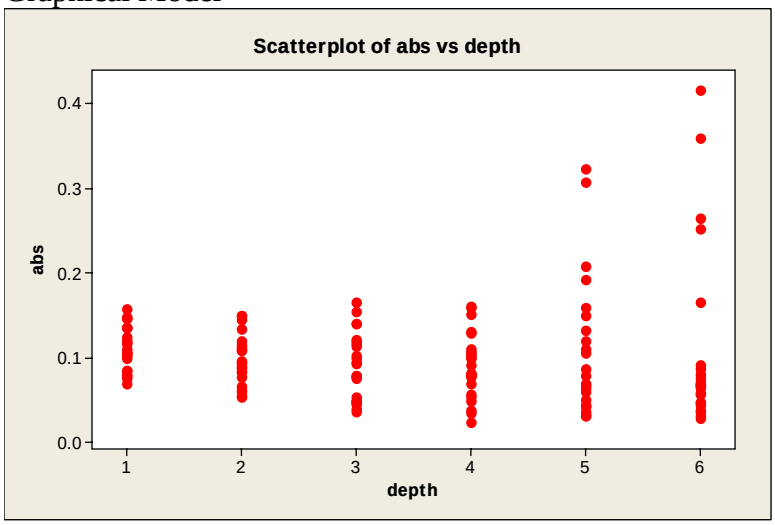
The p-values can be calculated from these numbers by subtracting the numbers obtained from the cdf command from 1. The p-values are as follows:

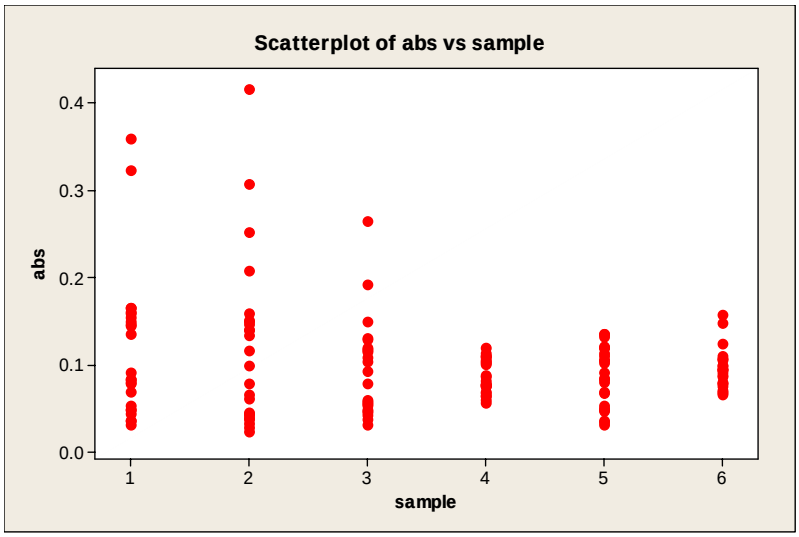
Elisa
p = 1-0.958626 = 0.0414

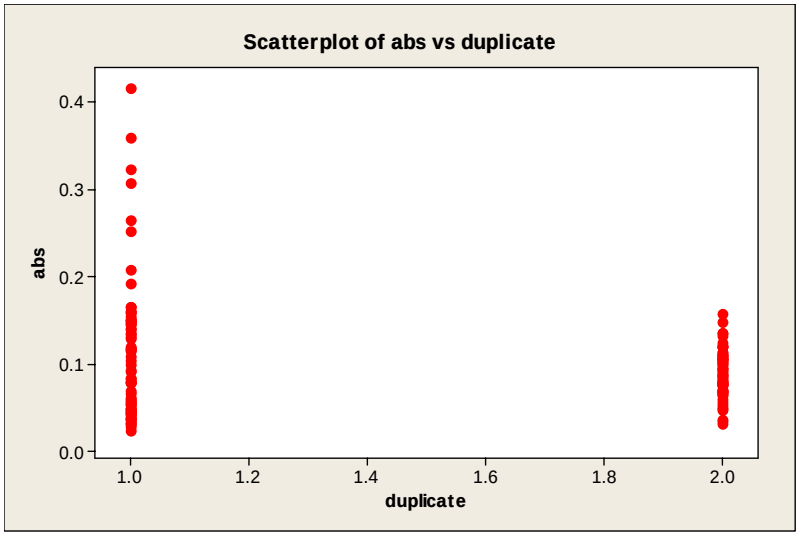
Depth
p = 1-0.0017729 = 0.998

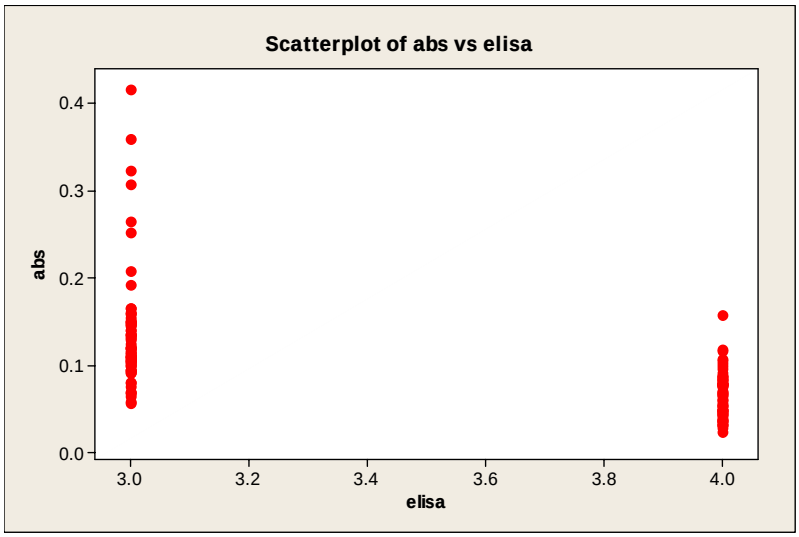
Elisa*depth
P = 1-0.0285272 = 0.971

Graphical Model

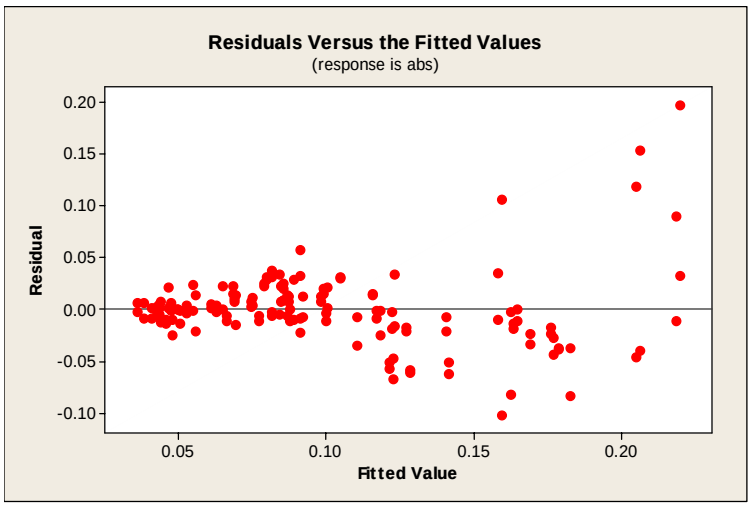



Scatterplot of abs vs sample
abs
0.0
0.1
0.2
0.3
0.4
1
2
3
4
5
6
sample

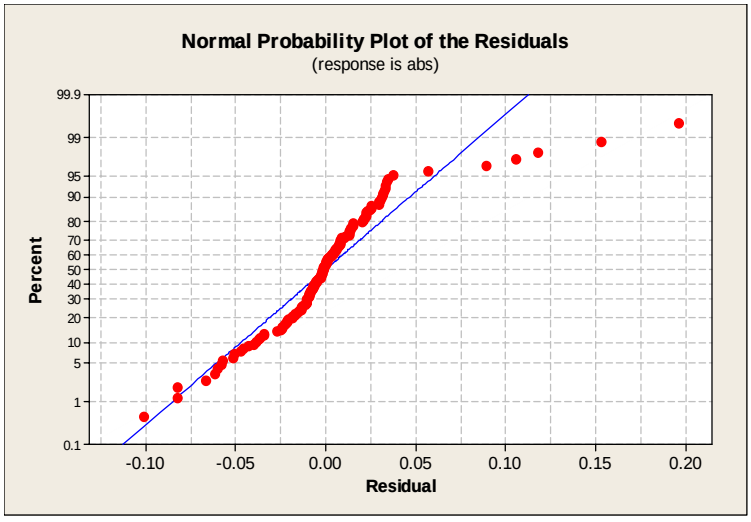

Scatterplot of abs vs duplicate
abs
0.0
0.1
0.2
0.3
0.4
1.0
1.2
1.4
1.6
1.8
2.0
duplicate

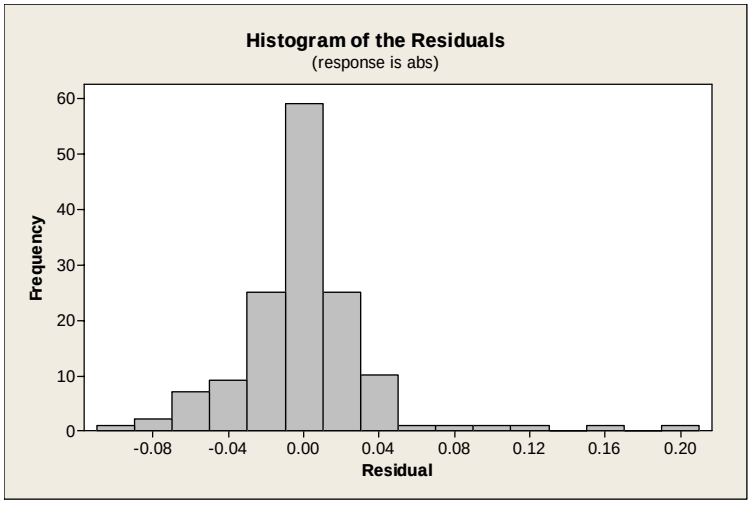

Scatterplot of abs vs elisa
abs
0.0
0.1
0.2
0.3
0.4
3.0
3.2
3.4
3.6
3.8
4.0
elisa

When the model was executed, the following diagnostic plots were produced.



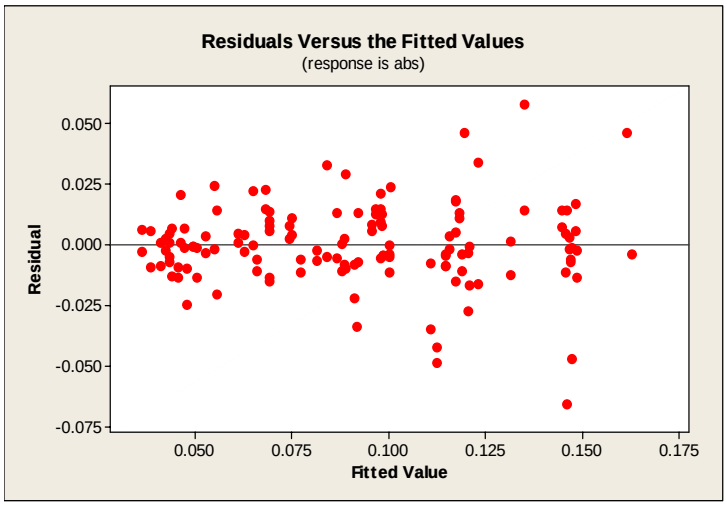

The residuals vs. Fits plot shows cones shaped patterns. Therefore the assumption cannot be met.

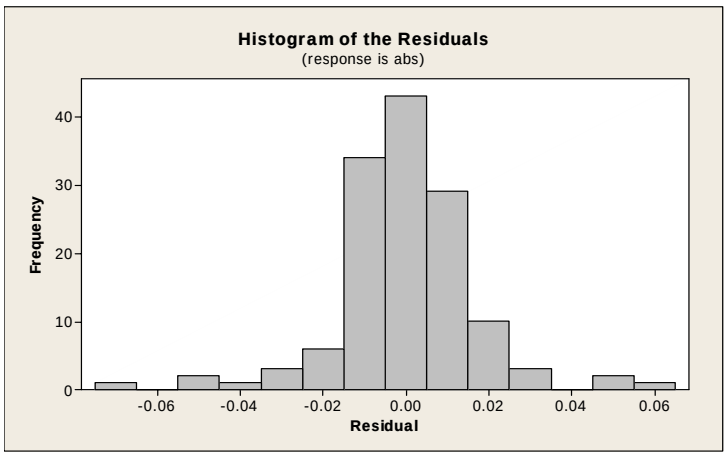


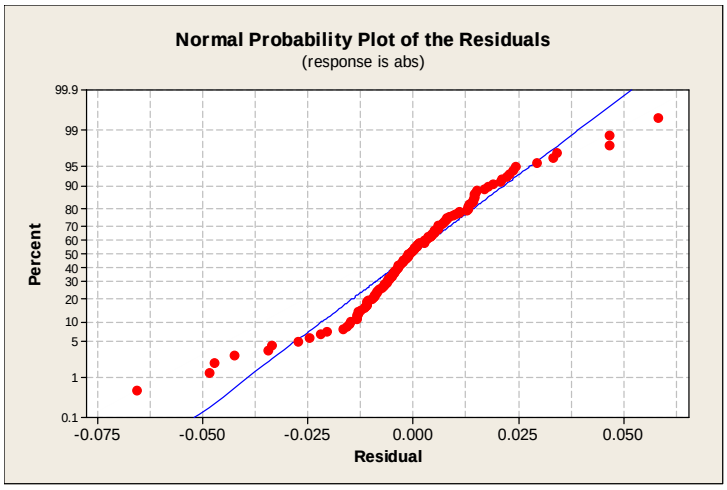



The normality plot and the histogram of residuals both show an that the data is not normal.

To deal with the problem of the data not being homogenous or normal, I removed any outlying data, ran the model again, and my new diagnostic plots were as follows:



Errors are now homogenous and closer to normal.

Since I removed some of my values, I had to recompute new MS's, F ratios and p-values. The new p-values are:

| Elisa | Depth | elisa*depth |
|---|---|---|
| p = 0.008 | p = 0.950 | p = 0.794 |

It can be seen that the interaction term, elisa*depth, is not found to be statistically significant, both with and without the outliers. This means that from this analysis, it can be determined that there was no significant difference in the location or depth of the mucin in the cesium chloride gradient between Elisa 3 and Elisa 4. However, it can be determined by analyzing the original data that differences in the positioning of the mucin between Elisa 3 and Elisa 4 was large. However, we see that this term cannot be defined as significant since the duplicate(elisa) term below it has a very large MS and F-ratio which will not allow the elisa*depth term to be significant. To obtain a better test, we would have to increase the number of replications performed in the experiment. This would decrease the variance of this variable and free up some effort to be placed in the other variables, including the elisa*depth term. This, however, is not appropriate in this situation since our lack of availability of appropriate equipment will not allow us to process this quantity of sample. By using this process, we would have excess sample, which would only be wasted since it would not fit into the centrifuge.

The $H_A/H_o$ pair for this set of data is:

Elisa $H_A$: var ($\beta_{elisa} \cdot$ elisa) > 0
$H_o$: var ($\beta_{elisa} \cdot$ elisa) = 0

Depth $H_A$: var ($\beta_{depth} \cdot$ depth) > 0
$H_o$: var ($\beta_{depth} \cdot$ depth) = 0

Elisa * Depth $H_A$: var ($\beta_{elisa*depth} \cdot$ elisa*depth) > 0
$H_o$: var ($\beta_{elisa*depth} \cdot$ elisa*depth) = 0

0.794 = p which is greater than 0.05. Therefore it can be determined that we can accept the $H_o$ that there is no interactive effect of Elisa 3 and 4 and the depth of the mucin on absorbance.

**Summary**

The statistical question of *whether or not there was consistency in the depth of the mucin location in the cesium chloride gradient* cannot be answered. Through a complicated analysis of my data, it was determined that there was no statistical difference in the position of mucin between Elisa 3 and 4, despite my anticipation that there was. It was interesting to see the effect that the large variation in the mucin content of duplicates in Elisa 3 had on the overall statistical analysis of my data. By undertaking this assignment and analyzing my data statistically, a greater understanding of the design of my project was learnt. As well, I now realize the importance of statistical analysis and the role of experimental design in allocating effort in order to detect differences.

## References


Claustre J, Toumi F, Trompette A, Jourdan G, Guignard H, Chayvialle JA, Plaisancie P (2002). Effects of peptides derived from dietary proteins on mucus secretion in rate jejunum. *Am. J. Physiol.* ***283*** *(Gastrointest. Liver Physiol*.): G521-528

Stoll, B, Burrin, DG, Henry, JF, Jahoor, F, Reeds, PJ (1999). Dietary and systemic phenylalanine utilization for mucosal and hepatic constitutive protein synthesis in pigs. *Am. J. Physiol.* ***276*** *(Gastrointest. Liver Physiol*. **39)**: G49-G57

Stoll, B, Henry, JF, Reeds, PJ, Yu, H, Jahoor, F, Burrin, DG (1998). Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. *J. Nutr.* **128**: 606-614

# Developing an elisa to pig small intestinal mucin

Submitted by: Natalie Nichols
Submitted on: December 3, 2004