The ABRF Edman Sequencing Research Group 2008 Study

Effects of a Homopolymeric amino acid tag on Edman Degradation
Current ESRG Members

- Richard S. Thoma (Co-Chair)  
  Monsanto

- Brian Hampton (Co-Chair)  
  University of Maryland School of Medicine

- Joseph W. Leone (Chair-emeritus)  
  Pfizer, Inc.

- Peter Hunziker  
  University of Zurich

- Klaus Linse  
  University of Texas - Austin

- Wendy Sandoval  
  Genentech, Inc.

- J. Steve Smith  
  University of Texas Medical Branch - Galveston

- Nancy D. Denslow (EB liaison)  
  University of Florida - Gainesville
What is the ESRG?

- **Mission of the Edman Sequencing Research Group:**

  “… to assist ABRF members in evaluating their capabilities to analyze the N-terminus of proteins/peptides using Edman Sequencing chemistry in order to establish realistic expectations for this technology. “

- **ESRG primary responsibility: a multi-lab participation educational study**
  - Sample Conception
  - Study Design (approval by EB)
  - Sample Preparation and distribution
  - Preliminary testing by group
  - Data collection, analysis and interpretation
  - Data presentation
    - Poster Presentation at ABRF meeting (Poster # RG8-M)
    - Oral Presentation at ABRF meeting
    - Publication in JBT (upcoming)
20 ESRG Studies: 1988-2008
A summary of past studies

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ESRG 2008 Study Objective

To determine the effects of a homo poly amino acid N-terminal tag on Edman degradation.

Are differences observed during Edman degradation between tagged proteins a consequence of repeating amino acids in general or is there something specific about Histidines that cause variable data?
Affinity Tags for Protein Purification

- Polypeptide sequences fused to recombinant proteins
- Allows the researcher to easily purify large amounts of recombinant protein
- Tags widely used in biotech industry
- Much variability in the type of tag, protein and purification system
Affinity Tags: General Properties

1. One-step absorption purification
2. Minimal effect on III$\text{o}$ Structure
3. Easy, specific removal to produce native protein
4. Simple assay of recombinant protein during purification
5. Wide applicability
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# Affinity Tags and N-terminal Sequence Analysis

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<td>Proper processing</td>
<td>Repeating Amino acids</td>
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<td>Difficult get N-termini from mass spec</td>
<td>Drop in yield of His tags</td>
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<td>Correct protein, open reading frame</td>
<td>Other proteins not having His tags bind Ni²⁺ (Albumin, endogenous proteins, SLYD_ECOLI)</td>
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Histidine Tags

- Histidines have strong interactions with immobilized metal ion matrices
- e⁻ donor on imidazole ring form coordination bonds with transition metals (ex. Ni²⁺, Co²⁺)
- 6xHis tags have a high affinity to Ni-NTA and so may be easily purified
- Proteins are eluted from matrix by adjusting pH or adding free imidazole
General Cloning and Expression Protocol: Adding a His Tag

1. Insert protein DNA into a vector encoding a His-tag

2. Perform PCR with primers that have repeating His codons next to start/stop

Modified from Wikipedia
Study Protein: Human Growth Hormone

- First recombinant pharmaceutical to be manufactured and marketed by a biotech company (1985 approval)

- 191 Amino acids
- 2 phosphorylation sites: Ser132, Ser176
- Not an ‘ideal’ protein for Edman
- Used as real life example

Sample Preparation

- Different homo-poly amino acid tags were grown on small scale (3 plates each)
- Some tags did not express even though constructs were correct!

Tags tried:

- **Phe** - A bulky AA (like His)
  - Good sequencability
- **Lys** - charged AA (like His)
- **Tyr** - A polar AA
- **Ala** - Small, reliable
ESRG samples Cloning, Expression, and Purification

1. Cloning
   - PCR
   - Digest and Ligate
     - Clai
     - Asci

2. Expression
   - Transfect into HEK 293 cells

3. Purification
   - Tagged and purified HGH protein
   - Ni$^{2+}$-IMAC

Jessica Huard
The samples...

Sample C:

FPTIPLSRLFDNAMLRA...

Sample H:

KHHHHHHHHHHLE FPTIPLSRLFDNAMLRA...

Sample A:

KIDAAAAAAA FPTIPLSRLFDNAMLRA...
Concentrations of His and Ala samples were estimated by control band intensity after coomassie staining.

~25 pmol of each sample (Control, His tag and Ala tag) were loaded onto SDS-PAGE gels and subsequently electroblotted onto PVDF.

Two excised bands from each of the 3 samples were sent to participating labs.
Participating Lab Instructions and Samples Sent out

October 31, 2007

To our colleagues,

Please find enclosed the ABRF2008 SSRR samples that you requested from the ABRF Event Sequence Research Group. This is the 20th study in an annual series designed to aid laboratories in evaluating their ability to obtain and interpret amino acid sequence data. This year, the test samples are three expressed proteins named A, B, and C spotted on PVDF membranes and stained with Coomassie Blue. For each protein you will receive two 1" x 1" pieces containing approximately 2500 mg of protein. Depending on the sensitivity of your instrument, you may load either one or two of the bands for the sequencing run. The SSRR recommends loading one band on the Proxeon C1 or 2 bands for the Proxeon HT.

The object of this year's study is to mimic the difficulty of sequencing through an informational density and to still be able to determine a few amino acids of the protein sequence following the gap, in order to be able to confirm the identity of the tagged samples. The SSRR is mailing all the participants in sequence 11 residues of each sample. If it is not possible for you to sequence all the samples we are asking to sequence at least sample A and B. Preferably, all data should be submitted in electronic form as text files created directly from the sequencing software. Note that you send us digital files only for each instrument in each cycle. On instruments connected to a Windows PC you can export the tabulated plot data (open under Y View, sequence data) as text file under “File -> Export”. On instruments connected to a Macintosh open the sequence data (Analyze -> Show Sequence Data), save the data as a text file (“File” menu select “Save As”).

The SSRR is asking participants to return the raw data text files and the survey sheet by email. You should have received an Excel file containing the survey sheet as attachment to the email confirmation of your sample request. If you did not receive the Excel file, or prefer a paper copy, please contact Brian Hopwood (bhopwood@merck.com) and he will send you a paper copy. The files with the results should be emailed to: Glenda Cowart (Glenda.Cowart@monsanto.com) as an attachment by December 15, 2007. If you are returning paper copies of the survey sheet and/or raw data text files on a disk-formatted floppy disc, please mail to:

Glenda Cowart
Monsanto Co.
1000 N. Lindbergh Blvd.
Mail Zone USA
St. Louis, MO 63147, USA

In order to ensure anonymity, Glenda Cowart will remove all identifying marks prior to forwarding the data to the sequence committee for analysis. An email will be sent to your facility with a three-digit code to allow you to evaluate your results as compared to others. The sequencing and survey results will be presented at a session on February 4-15, 2008 in Salt Lake City, Utah, and will also help to guide future potential studies and lab tests.

If you have any questions about the study or have problems with the data, please contact Brian Hopwood at the above email address. Equipment failures and/or data obtained analysis are as important to us as data from successful runs. Please send us your results whenever they happen. Thank you for your participation in this study.

The deadline for receiving data for inclusion in the study is December 1, 2007.

The editorial team of Sequencing Research Group:

Richard Thome (Chair), Brian Hopwood (Cochair), Joe Leone
Monsanto, Univ. of Maryland, Pfizer

Wendy Barlow, Klaus Umm, Peter Wassertein, Steve Smith
Cerental, Univ. of Texas, Univ. of Zurich, Univ. of Texas

Nancy Schimovitz (Secretary)
University of Florida

21 labs received samples
The Results
Results: Instrumentation and Methods Survey

Information Requested:

- Sequence calls
- Instrument type (cLC or HT?)
- Reagent information
- Standard RT and areas
- Column Information
- Gradient
Results:
Uncorrected Raw Data Returned in Excel Format

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</tbody>
</table>
Participating Lab Information (n=21)

Manufacturer and Model:  
13 ABI 494 HT (1-11 years old: average age 8 years)  
6 ABI 492 cLC (5-11 years old: average age 7-8 years)  
2 ABI 494 cLC (6-11 years old: average age 7 years)  
1 ABI 491

Reagents:  
18 used all instrument manufacturer reagents  
3 used some mfg. R1, R2c, R4, R5, & Premix  
1 said almost mfg. reagents

TFA Cleavage:  
19 used pulsed liquid, 3 gas phase

Chemistry Cycle:  
16 PVDF, 3 GFF, 1 PVDF & Prosorb, 1 pulsed Liq cLC

Other Additives:  
1 TCEP to R4 & R5, 1 DTT in R4 & 1 DTT in S2.  
1 n-acetylcysteine in R5, 1 34% Me-Pip, 52%IPA in R2

Bands Loaded:  
9 x 1; 12 x 2

Columns Used:  
11 ABI Spherisorb 5 micron PTH column (2.1 x 220 mm)  
1 Higgins column 3 micron (2.1 x 100)  
1 Higgins column 5 micron (2.1 x 220)  
8 ABI Prosice cLC PTH column 5 micron (0.8 x 250)

Typical Gradients used:

<table>
<thead>
<tr>
<th>HPLC Gradient cLC</th>
<th>Time</th>
<th>% B</th>
<th>Flow Rate (µl/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>23</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>44</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>95</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>95</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HPLC Gradient HT</th>
<th>Time</th>
<th>% B</th>
<th>Flow Rate (µl/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>8</td>
<td>325</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>8</td>
<td>325</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>23</td>
<td>325</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>44</td>
<td>325</td>
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</tr>
<tr>
<td></td>
<td>26</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>
Sequence calls and average lag (in red)

Sample H: KHHHHHHHHHEL EFP TIP L
Sample A: KI DAAAAAA AAF P TIP L
Sample C: FPTIP LSRLFLDFN AM LRA
Initial Yields
Repetitive Yields

\[
RY = \sqrt[\text{x}]{\frac{\text{(pmol AA)}_{n+x}}{\text{(pmol AA)}_n}}
\]

Average repetitive yield (%)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample C</td>
<td>90.00</td>
</tr>
<tr>
<td>Sample H</td>
<td>100.00</td>
</tr>
<tr>
<td>Sample A</td>
<td>90.00</td>
</tr>
</tbody>
</table>
Average Recoveries for His and Ala Tagged Samples

Mean Values for His and Ala for All Laboratories

Normalized Mean Yields (picomoles) vs. Cycle Number
Lag at Phe

Sample C: F-P-T-I-P-L-S-R-L-F-D-N-A-M-L-R-A
Lag overtaking yield

Average Cycle where Lag Overtakes Yield

![Bar chart showing the average cycle where lag overtakes yield for Sample H and Sample A.](chart.png)
Phenylalanine Yields Throughout Run

Yield of Phe in All Cycles

- Sample H
- Sample A
- Sample C
Decrease in pmol recovery after first cycle

Ratio of Cycle One to first Cycle of Tag

Sample H – Cycle 1
Sample H – Cycle 2
Sample A – Cycle 1
Sample A – Cycle 4
Conclusions

- Creation of a poly-amino acid is not easy to do. Other than the traditional His-tag, the only other successfully prepared poly amino acid tag for this study was an Ala tag.
- There was a noticeable decrease in initial yields of the His samples versus controls.
- Labs in general found it harder to call the sequence after the poly-His tag than the other two samples.
- Lag was observed earlier and more consistently on the His tagged sample than the Ala tag.
- High variability is due to lab to lab variability.
- The majority of labs successfully sequenced seventeen cycles for all three test proteins.
Acknowledgements

- **Jessica Huard** - Genentech
  - Expression and Purification of study samples

- **Liza Ingle** - Genentech
  - Running gels, blots and sequencing for initial analysis and distribution

- **Glenda Cowart** – Monsanto
  - Accumulation of data

- **Participating labs**

- **Executive Board**
  - For support and scrutiny of study proposal

- **ESRG members**
  - Design and execution of study