# Modifications

MASCOT

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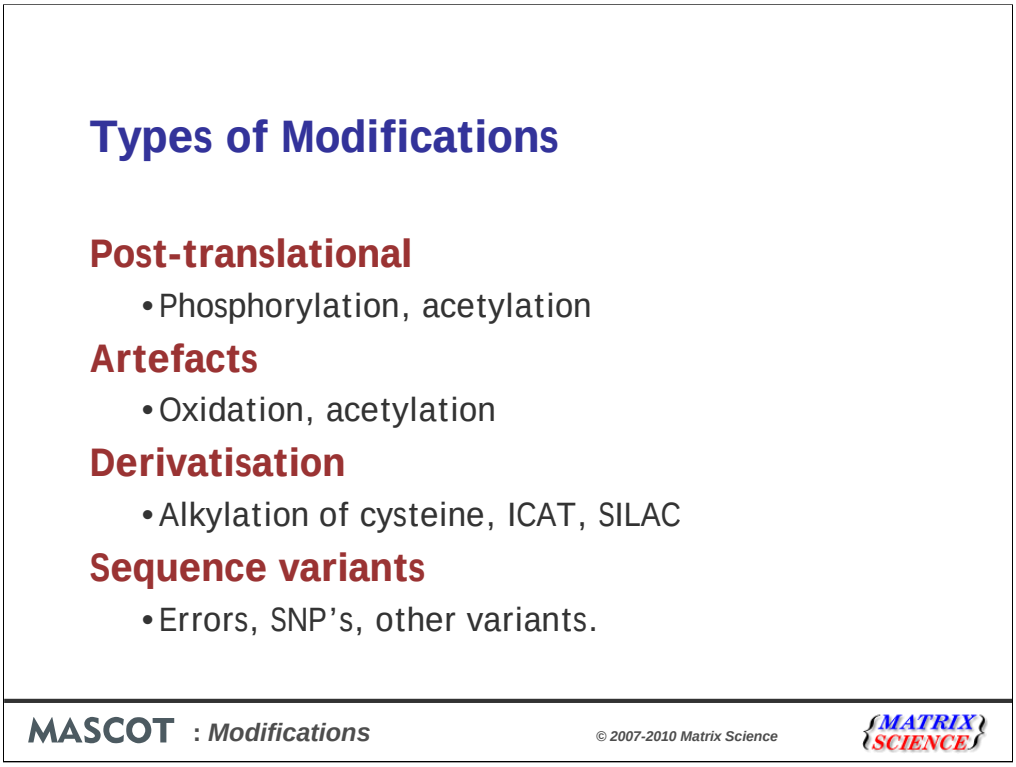


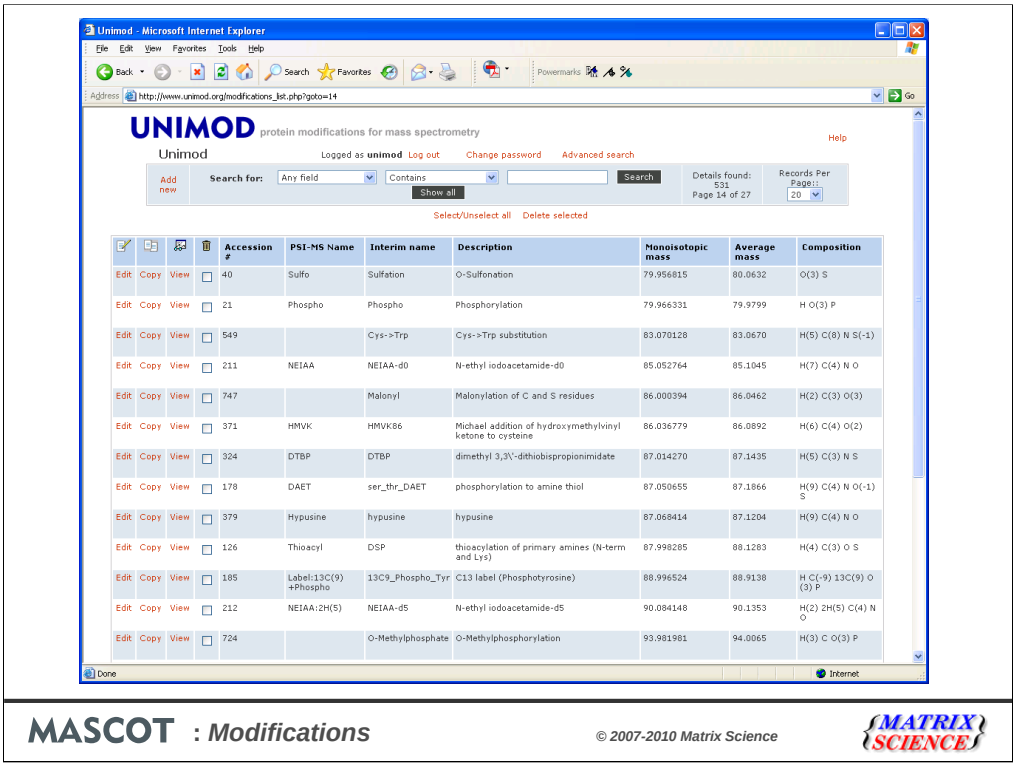

Modifications are a very important topic in database searching.

In some cases, the main focus of a study is to characterise post translational modifications, which may have biological significance. Phosphorylation would be a good example.

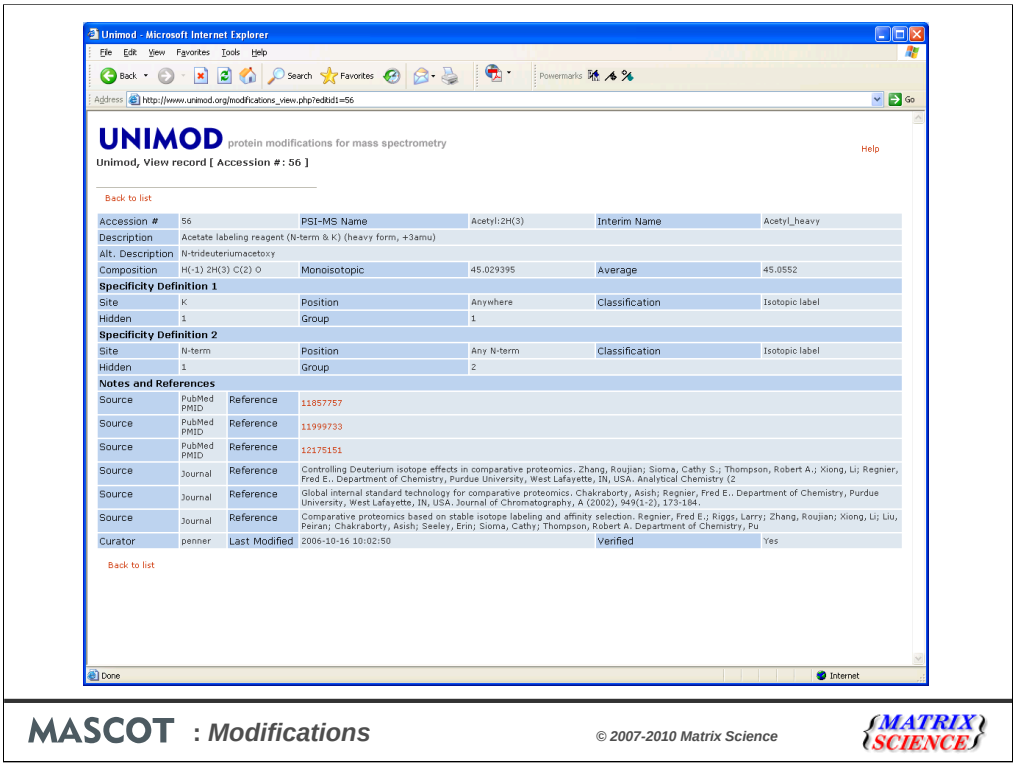
In other cases, the modification may not be of interest in itself, but you need to allow for it in order to get a match. Oxidation during sample preparation would be an example.

And, of course, most methods of quantitation involve tagging

Some sequence variants, such as the substitution of one residue by another, are equivalent to modifications, and can be handled in a similar way

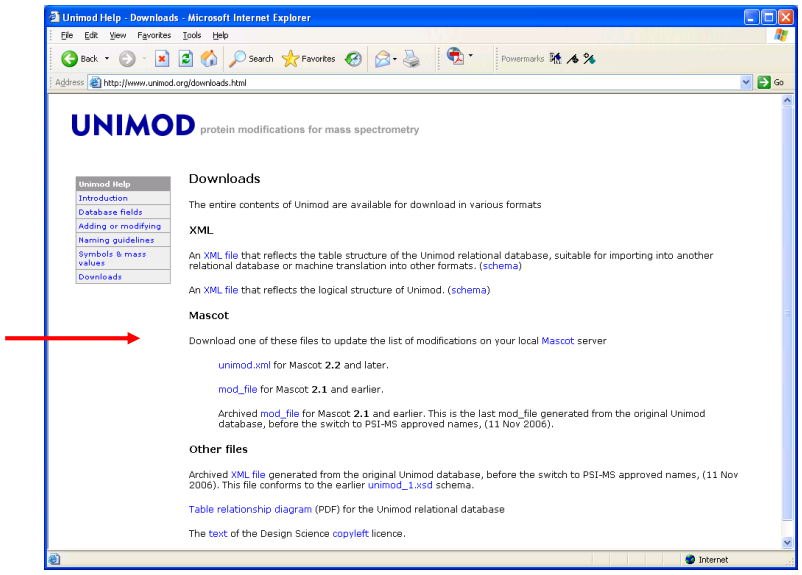



Comprehensive and accurate information about post translational and chemical modifications is an essential factor in the success of protein identification. In Mascot, we take our list of modifications from Unimod, which is an on-line modifications database.




There are other lists of modifications on the web, like DeltaMass on the ABRF web site and RESID from the EBI, but none is as comprehensive as Unimod

Mass values are calculated from empirical chemical formulae, eliminating the most common source of error. Specificities can be defined in ways that are useful in database searching, and there is the option to enter mass-spec specific data, such as neutral loss information. This screen shot shows one of the better annotated entries, I can't pretend that all of them are this detailed. Nevertheless, it is a very useful, public domain resource that beats having to create your own list in an Excel spreadsheet or on the back of an envelope.

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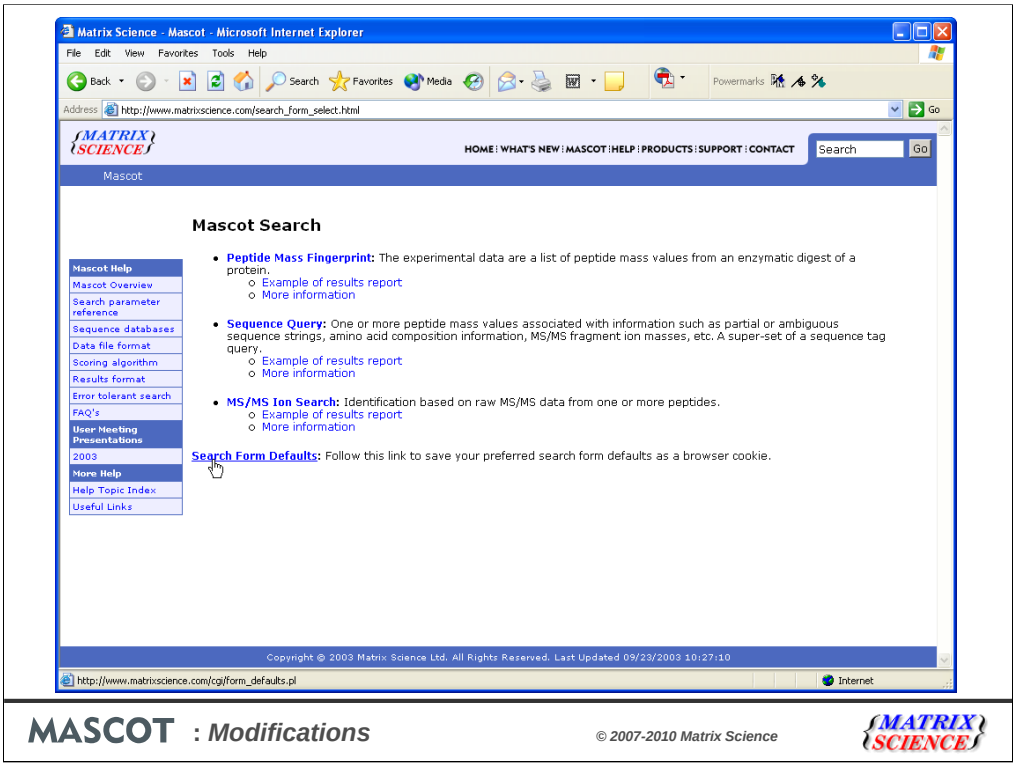
If you go to the help page, there is a link to download the contents of Unimod as a Mascot modifications file. This is the easiest way to keep the modifications list on an in-house Mascot server up-to-date

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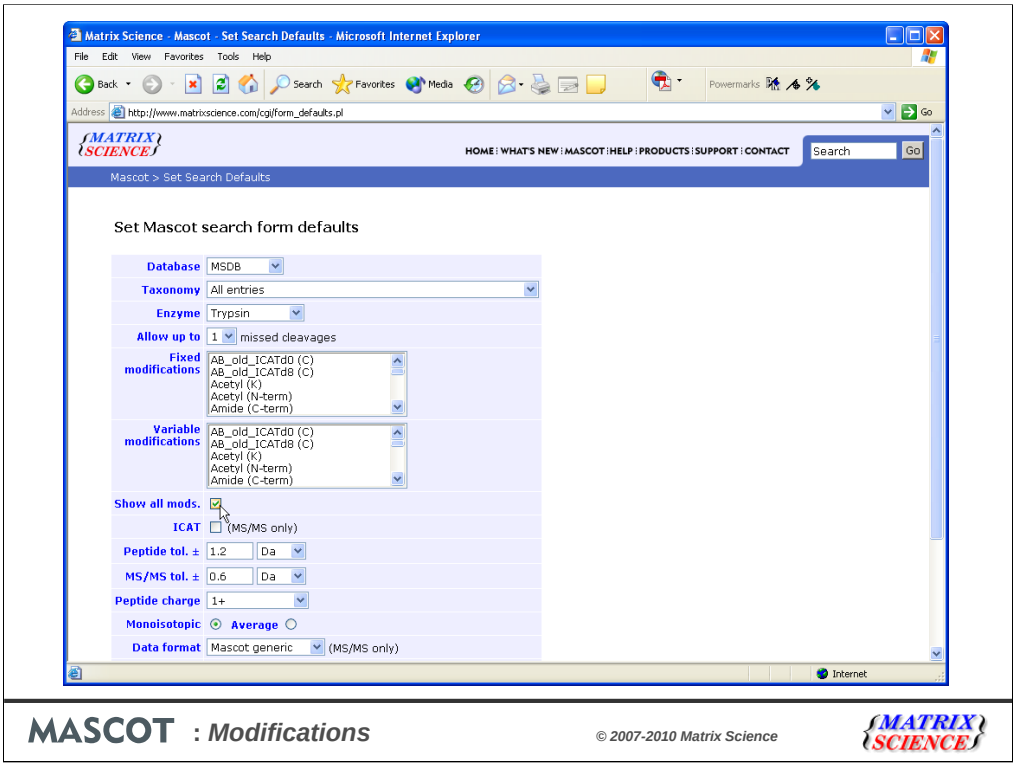
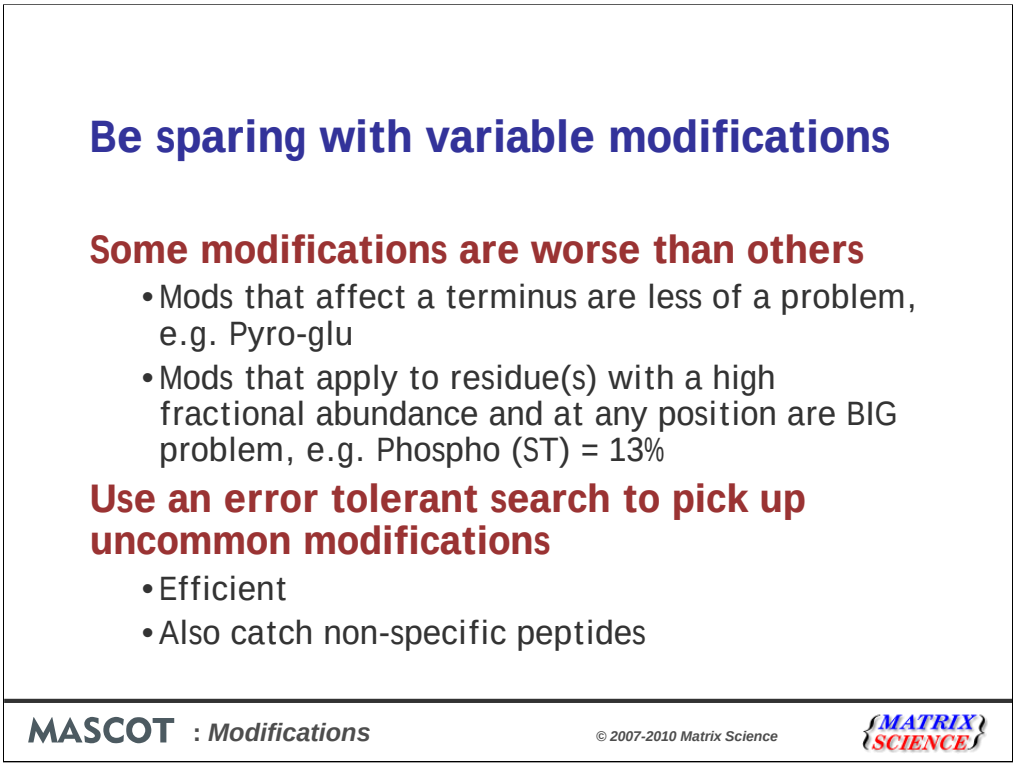
Here is a tip. The default list of modifications displayed in the Mascot search form is a short list, containing only the most common mods. If you want to see the complete list of mods, and you are using Mascot 2.2 or earlier, you need to follow the link at the bottom of the search form selection page







Check the box for Show all mods, then choose Save. This still sets the default state of the checkbox in Mascot 2.3, but we decided to place the checkbox on the search form, so as to make it easier to swap between the short and long lists.



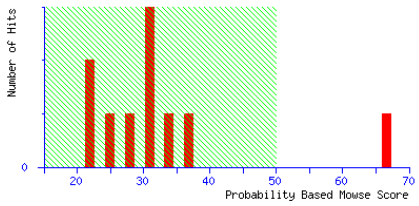

It is extremely important that you do not choose more than the absolute minimum number of variable modification in a search. We talked about this in an earlier presentation, but it is worth repeating.

Variable or differential or non-quantitative modifications are expensive, in the sense that they increase the time taken for a search and reduce its specificity. This is because the software has to permute out all the possible arrangements of modified and unmodified residues that fit to the peptide molecular mass. As more and more modifications are considered, the number of combinations and permutations increases geometrically. The so-called combinatorial explosion.

Some variable modifications are worse than others. Modifications that only apply to a terminus, especially if they only apply when particular residue is at the terminus, like pyro-glu, make little difference to the number of peptides to be tested. The problem modifications are the ones that apply to residues in any position, especially if they apply to multiple residues, like phosphorylation.

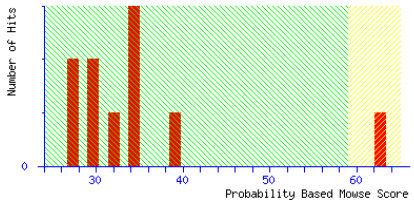
Unless you have enriched the sample in a particular PT-mod, e.g IMAC for phosphopeptides, it is usually not a good idea to try and catch PT-mods in a first pass search. Better to use a second pass search, which we call an error tolerant search, to catch the low abundance mods. We will come back to this later.

## Be sparing with variable modifications




Oxidation (M)

**8 sec**




Acetyl (K)
Carbamidomethyl (C)
Carboxymethyl (C)
Me-ester (DE)
Oxidation (M)
Phospho (ST)
Phospho (Y)
Sodiated (DE)

**92 sec**

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To illustrate this point. This search of a single MS/MS spectrum, using one variable mod, gives a nice, statistically significant match.

If the search is repeated with 8 mods, the match is the same, with an identical score, but now it is barely significant.

All of these mods have effectively increased the size of the database by a factor of 30

What's worse, the search takes over 10 times as long!

So, use variable mods sparingly. You'll get better results and faster.

By the way, the yellow region in the histogram indicates scores above the homology but below the identity thresholds. You will only see these regions highlighted in an MS/MS search report if it is a search of a single spectrum.

## Why is phosphorylation such a challenge?

**Site heterogeneity**

**Poor ionisation efficiency**

**3 fragmentation channels**

- intact fragments
- neutral loss of $HPO_3$ (80 Da)
- neutral loss of $H_3PO_4$ (98 Da)

**Can occur at STY - ~16% of residues.**

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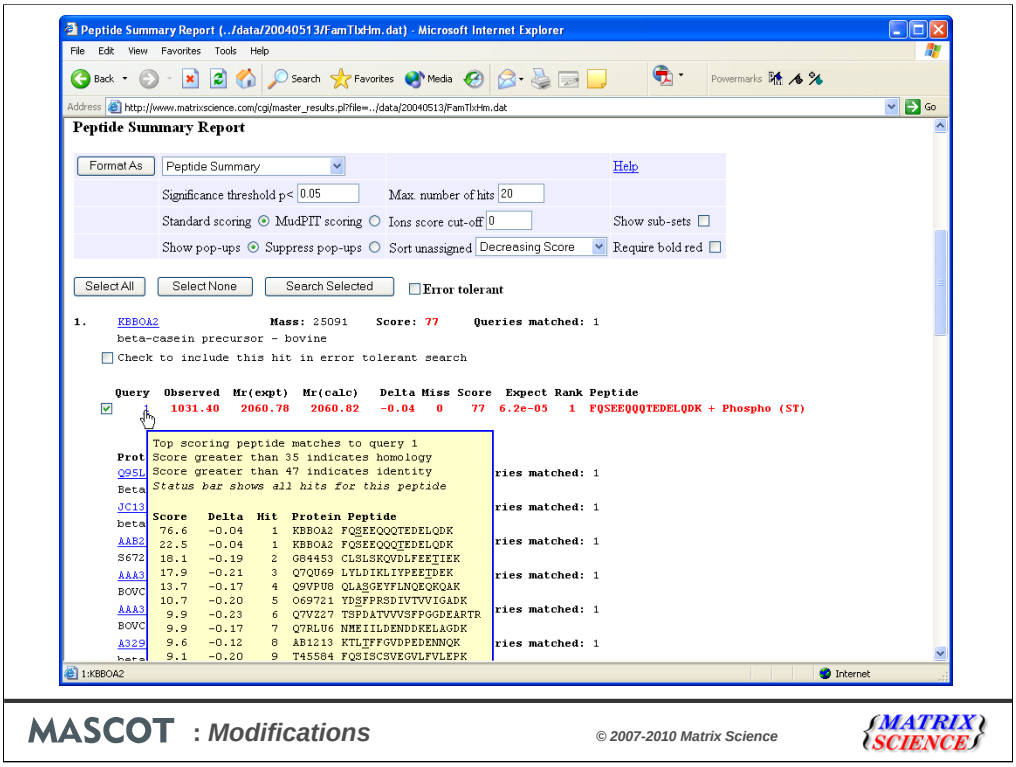
Of all post-translational modifications, phosphorylation is one of the most interesting and also one of the most difficult. Why is it such a challenge?

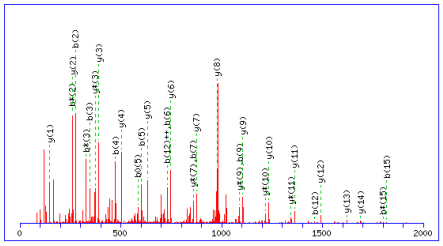







Lets look at an example or two.

One of the most common phosphopeptides comes from the milk protein, beta casein. There are two potential phosphorylation sites, S and T, but only one is modified. Because the two sites are widely separated, there is no ambiguity, even if the spectrum is not the greatest.




**Monoisotopic mass of neutral peptide Mr(calc):** 2060.82
**Variable modifications:**
**S3** : Phospho (ST)
**Ions Score:** 77 **Expect:** 4e-05
**Matches (Bold Red):** 33/172 fragment ions using 65 most intense peaks

| # | b | $b^{++}$ | $b^*$ | $b^{*++}$ | $b^0$ | $b^{0++}$ | Seq. | y | $y^{++}$ | $y^*$ | $y^{*++}$ | $y^0$ | $y^{0++}$ | # |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 148.08 | 74.54 | | | | | F | | | | | | | 16 |
| 2 | **276.13** | 138.57 | **259.11** | 130.06 | | | Q | 1816.78 | 908.90 | 1799.76 | 900.38 | 1798.77 | 899.89 | 15 |
| 3 | **345.16** | 173.08 | **328.13** | 164.57 | 327.15 | 164.08 | S | **1688.72** | 844.87 | 1671.70 | 836.35 | 1670.71 | 835.86 | 14 |
| 4 | **474.20** | 237.60 | 457.17 | 229.09 | 456.19 | 228.60 | E | **1619.70** | 810.36 | 1602.68 | 801.84 | 1601.69 | 801.35 | 13 |
| 5 | **603.24** | 302.12 | 586.21 | 293.61 | **585.23** | 293.12 | E | **1490.66** | 745.83 | 1473.63 | 737.32 | 1472.65 | 736.83 | 12 |
| 6 | **731.30** | 366.15 | 714.27 | 357.64 | 713.29 | 357.15 | Q | **1361.62** | 681.31 | **1344.59** | 672.80 | 1343.61 | 672.31 | 11 |
| 7 | **859.36** | 430.18 | 842.33 | 421.67 | 841.35 | 421.18 | Q | **1233.56** | 617.28 | **1216.53** | 608.77 | 1215.55 | 608.28 | 10 |
| 8 | 987.42 | 494.21 | 970.39 | 485.70 | 969.41 | 485.21 | Q | **1105.50** | 553.25 | **1088.47** | 544.74 | 1087.49 | 544.25 | 9 |
| 9 | **1088.46** | 544.74 | 1071.44 | 536.22 | 1070.45 | 535.73 | T | **977.44** | 489.22 | 960.42 | 480.71 | 959.43 | 480.22 | 8 |
| 10 | 1217.51 | 609.26 | 1200.48 | 600.74 | 1199.50 | 600.25 | E | **876.39** | 438.70 | **859.37** | 430.19 | 858.38 | 429.70 | 7 |
| 11 | 1332.53 | 666.77 | 1315.51 | 658.26 | 1314.52 | 657.77 | D | **747.35** | 374.18 | 730.33 | 365.67 | 729.34 | 365.17 | 6 |
| 12 | **1461.58** | **731.29** | 1444.55 | 722.78 | 1443.57 | 722.29 | E | **632.32** | 316.67 | 615.30 | 308.15 | 614.31 | 307.66 | 5 |
| 13 | 1574.66 | 787.83 | 1557.63 | 779.32 | 1556.65 | 778.83 | L | **503.28** | 252.14 | 486.26 | 243.63 | 485.27 | 243.14 | 4 |
| 14 | 1702.72 | 851.86 | 1685.69 | 843.35 | 1684.71 | 842.86 | Q | **390.20** | 195.60 | **373.17** | 187.09 | 372.19 | 186.60 | 3 |
| 15 | **1817.75** | 909.38 | **1800.72** | 900.86 | 1799.74 | 900.37 | D | **262.14** | 131.57 | 245.11 | 123.06 | 244.13 | 122.57 | 2 |
| 16 | | | | | | | K | **147.11** | 74.06 | 130.09 | 65.55 | | | 1 |

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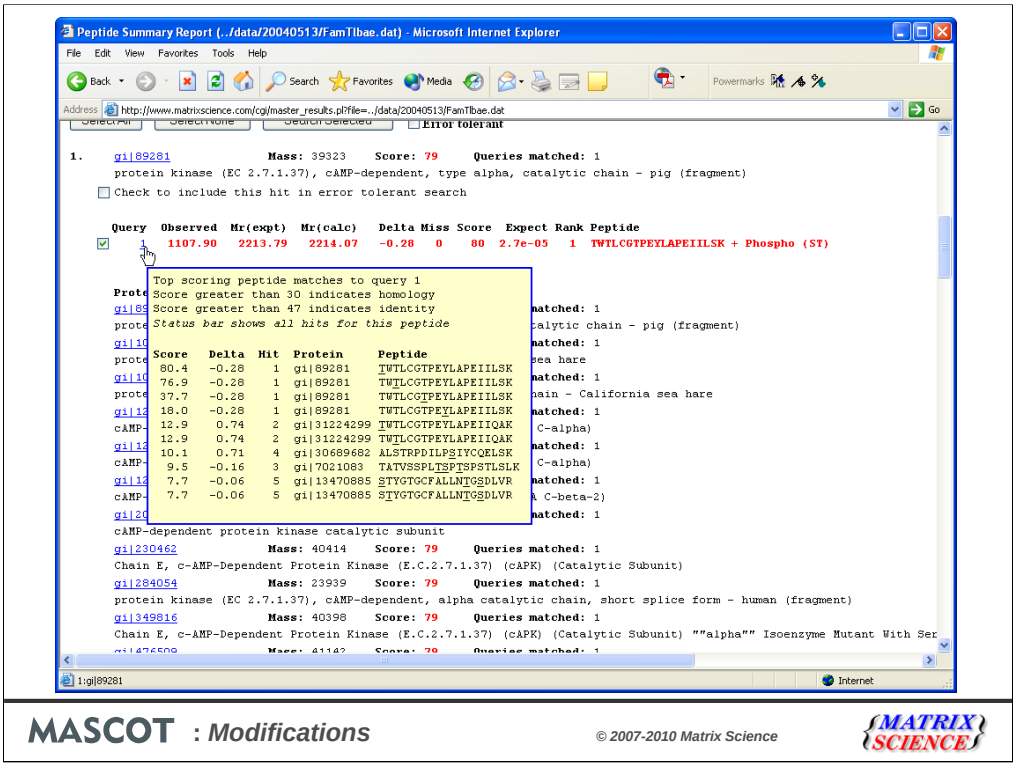
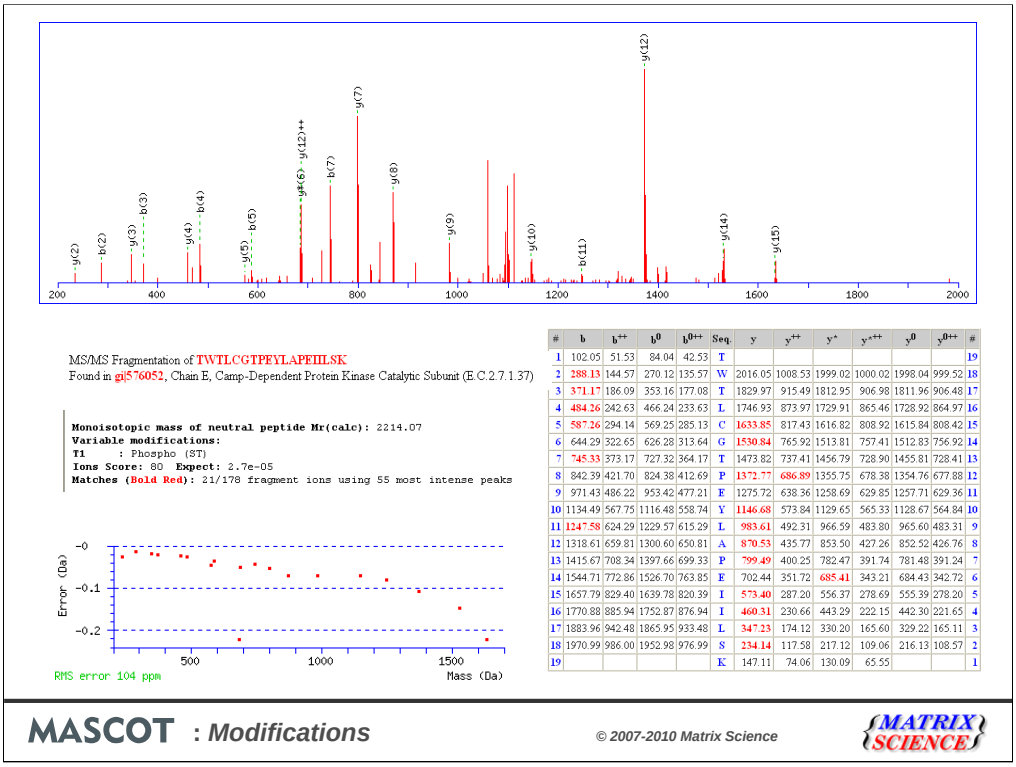
Beautiful spectrum; long run of y ions; move site to T9 and many matches would disappear



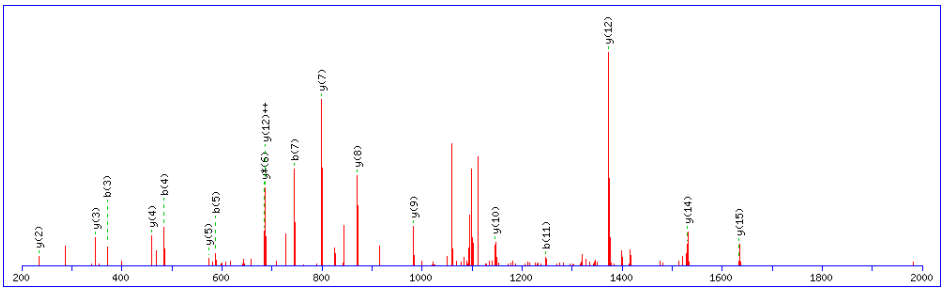

However, casein peptides are unusually easy to analyse. Here is a more typical example of what you can expect to find - a strong match to a phosphopeptide from a protein kinase.

There is little to choose in terms of score between having the phosphate on T1 or T3. We just can't say which site is modified, or whether there is a mixture of both isoforms. But, we can be very confident it is not on T7 or Y10 because the score drops dramatically



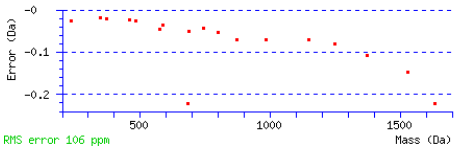

| # | b | b++ | b0 | b0++ | Seq. | y | y++ | y* | y*++ | y0 | y0++ | # |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 102.05 | 51.53 | 84.04 | 42.53 | T | | | | | | | 19 |
| 2 | 288.13 | 144.57 | 270.12 | 135.57 | W | 2016.05 | 1008.53 | 1999.02 | 1000.02 | 1998.04 | 999.52 | 18 |
| 3 | 371.17 | 186.09 | 353.16 | 177.08 | T | 1829.97 | 915.49 | 1812.95 | 906.98 | 1811.96 | 906.48 | 17 |
| 4 | 484.26 | 242.63 | 466.24 | 233.63 | L | 1746.93 | 873.97 | 1729.91 | 865.46 | 1728.92 | 864.97 | 16 |
| 5 | 587.26 | 294.14 | 569.25 | 285.13 | C | 1633.85 | 817.43 | 1616.82 | 808.92 | 1615.84 | 808.42 | 15 |
| 6 | 644.29 | 322.65 | 626.28 | 313.64 | G | 1530.84 | 765.92 | 1513.81 | 757.41 | 1512.83 | 756.92 | 14 |
| 7 | 745.33 | 373.17 | 727.32 | 364.17 | T | 1473.82 | 737.41 | 1456.79 | 728.90 | 1455.81 | 728.41 | 13 |
| 8 | 842.39 | 421.70 | 824.38 | 412.69 | P | 1372.77 | 686.89 | 1355.75 | 678.38 | 1354.76 | 677.88 | 12 |
| 9 | 971.43 | 486.22 | 953.42 | 477.21 | E | 1275.72 | 638.36 | 1258.69 | 629.85 | 1257.71 | 629.36 | 11 |
| 10 | 1134.49 | 567.75 | 1116.48 | 558.74 | Y | 1146.68 | 573.84 | 1129.65 | 565.33 | 1128.67 | 564.84 | 10 |
| 11 | 1247.58 | 624.29 | 1229.57 | 615.29 | L | 983.61 | 492.31 | 966.59 | 483.80 | 965.60 | 483.31 | 9 |
| 12 | 1318.61 | 659.81 | 1300.60 | 650.81 | A | 870.53 | 435.77 | 853.50 | 427.26 | 852.52 | 426.76 | 8 |
| 13 | 1415.67 | 708.34 | 1397.66 | 699.33 | P | 799.49 | 400.25 | 782.47 | 391.74 | 781.48 | 391.24 | 7 |
| 14 | 1544.71 | 772.86 | 1526.70 | 763.85 | E | 702.44 | 351.72 | 685.41 | 343.21 | 684.43 | 342.72 | 6 |
| 15 | 1657.79 | 829.40 | 1639.78 | 820.39 | I | 573.40 | 287.20 | 556.37 | 278.69 | 555.39 | 278.20 | 5 |
| 16 | 1770.88 | 885.94 | 1752.87 | 876.94 | I | 460.31 | 230.66 | 443.29 | 222.15 | 442.30 | 221.65 | 4 |
| 17 | 1883.96 | 942.48 | 1865.95 | 933.48 | L | 347.23 | 174.12 | 330.20 | 165.60 | 329.22 | 165.11 | 3 |
| 18 | 1970.99 | 986.00 | 1952.98 | 976.99 | S | 234.14 | 117.58 | 217.12 | 109.06 | 216.13 | 108.57 | 2 |
| 19 | | | | | K | 147.11 | 74.06 | 130.09 | 65.55 | | | 1 |

We can see why there is little difference in score between placing the phosphate on T1 or T3. There is just one extra matched peak, and in probability terms, there isn't a huge difference between 20 matches using 55 experimental peaks and 21. However, if you had to choose one or the other, you'd probably go for T1




MS/MS Fragmentation of TWTLCGTPEYLAPEIILSK
Found in gi|576052, Chain E, Camp-Dependent Protein Kinase Catalytic Subunit (E.C.2.7.1.37)

**Monoisotopic mass of neutral peptide Mr(calc):** 2214.07
**Variable modifications:**
**T3** : Phospho (ST)
**Ions Score:** 77 **Expect:** 6.1e-05
**Matches (Bold Red):** 20/178 fragment ions using 55 most intense peaks




| # | b | $b^{++}$ | $b^0$ | $b^{0++}$ | Seq. | y | $y^{++}$ | $y^*$ | $y^{*++}$ | $y^0$ | $y^{0++}$ | # |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 84.04 | 42.53 | 66.03 | 33.52 | T | | | | | | | 19 |
| 2 | 270.12 | 135.57 | 252.11 | 126.56 | W | 2034.06 | 1017.53 | 2017.04 | 1009.02 | 2016.05 | 1008.53 | 18 |
| 3 | **371.17** | 186.09 | 353.16 | 177.08 | T | 1847.98 | 924.49 | 1830.96 | 915.98 | 1829.97 | 915.49 | 17 |
| 4 | **484.26** | 242.63 | 466.24 | 233.63 | L | 1746.93 | 873.97 | 1729.91 | 865.46 | 1728.92 | 864.97 | 16 |
| 5 | **587.26** | 294.14 | 569.25 | 285.13 | C | **1633.85** | 817.43 | 1616.82 | 808.92 | 1615.84 | 808.42 | 15 |
| 6 | 644.29 | 322.65 | 626.28 | 313.64 | G | **1530.84** | 765.92 | 1513.81 | 757.41 | 1512.83 | 756.92 | 14 |
| 7 | **745.33** | 373.17 | 727.32 | 364.17 | T | 1473.82 | 737.41 | 1456.79 | 728.90 | 1455.81 | 728.41 | 13 |
| 8 | 842.39 | 421.70 | 824.38 | 412.69 | P | **1372.77** | **686.89** | 1355.75 | 678.38 | 1354.76 | 677.88 | 12 |
| 9 | 971.43 | 486.22 | 953.42 | 477.21 | E | 1275.72 | 638.36 | 1258.69 | 629.85 | 1257.71 | 629.36 | 11 |
| 10 | 1134.49 | 567.75 | 1116.48 | 558.74 | Y | **1146.68** | 573.84 | 1129.65 | 565.33 | 1128.67 | 564.84 | 10 |
| 11 | **1247.58** | 624.29 | 1229.57 | 615.29 | L | **983.61** | 492.31 | 966.59 | 483.80 | 965.60 | 483.31 | 9 |
| 12 | 1318.61 | 659.81 | 1300.60 | 650.81 | A | **870.53** | 435.77 | 853.50 | 427.26 | 852.52 | 426.76 | 8 |
| 13 | 1415.67 | 708.34 | 1397.66 | 699.33 | P | **799.49** | 400.25 | 782.47 | 391.74 | 781.48 | 391.24 | 7 |
| 14 | 1544.71 | 772.86 | 1526.70 | 763.85 | E | 702.44 | 351.72 | **685.41** | 343.21 | 684.43 | 342.72 | 6 |
| 15 | 1657.79 | 829.40 | 1639.78 | 820.39 | I | **573.40** | 287.20 | 556.37 | 278.69 | 555.39 | 278.20 | 5 |
| 16 | 1770.88 | 885.94 | 1752.87 | 876.94 | I | **460.31** | 230.66 | 443.29 | 222.15 | 442.30 | 221.65 | 4 |
| 17 | 1883.96 | 942.48 | 1865.95 | 933.48 | L | **347.23** | 174.12 | 330.20 | 165.60 | 329.22 | 165.11 | 3 |
| 18 | 1970.99 | 986.00 | 1952.98 | 976.99 | S | **234.14** | 117.58 | 217.12 | 109.06 | 216.13 | 108.57 | 2 |
| 19 | | | | | K | 147.11 | 74.06 | 130.09 | 65.55 | | | 1 |

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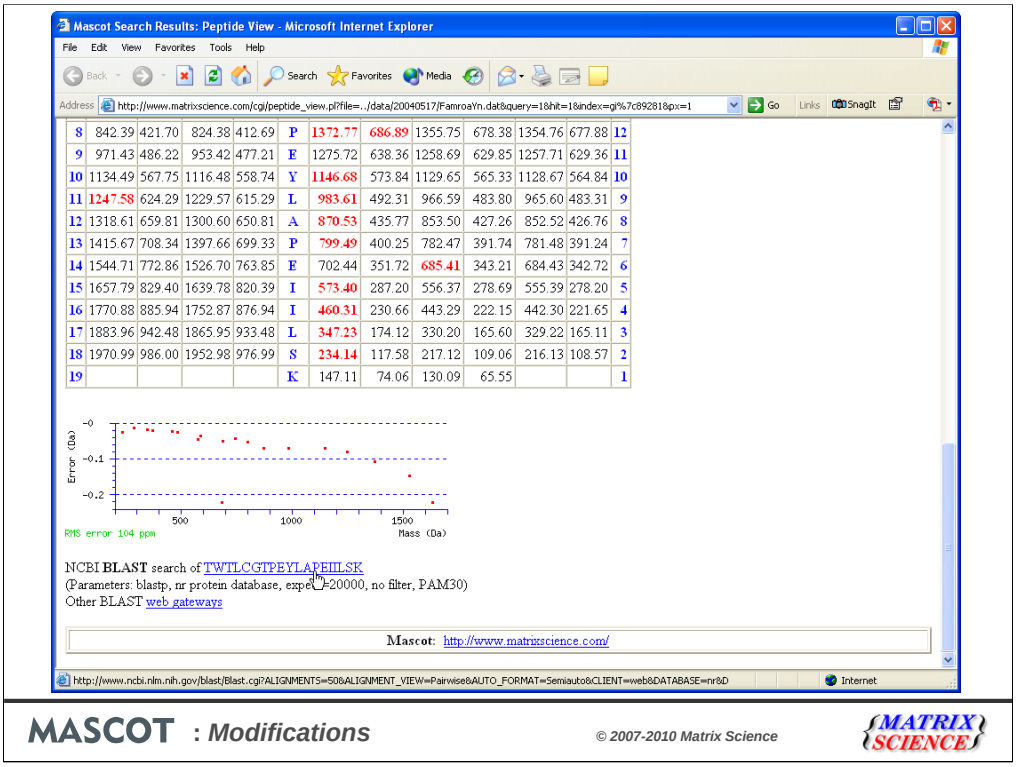
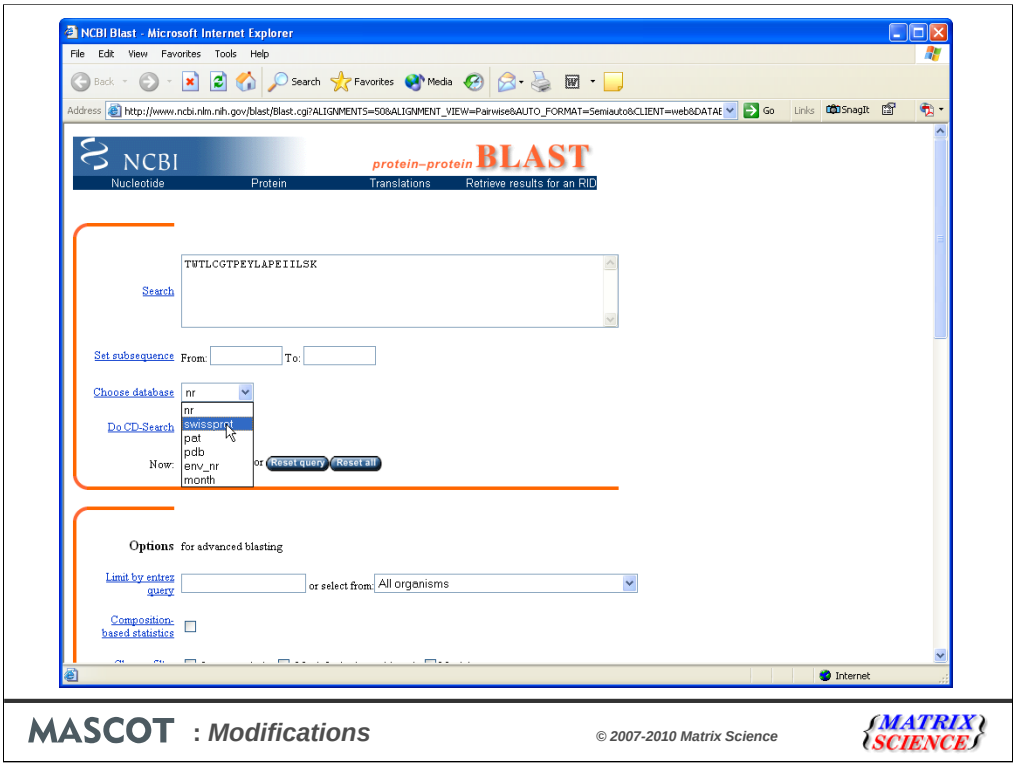


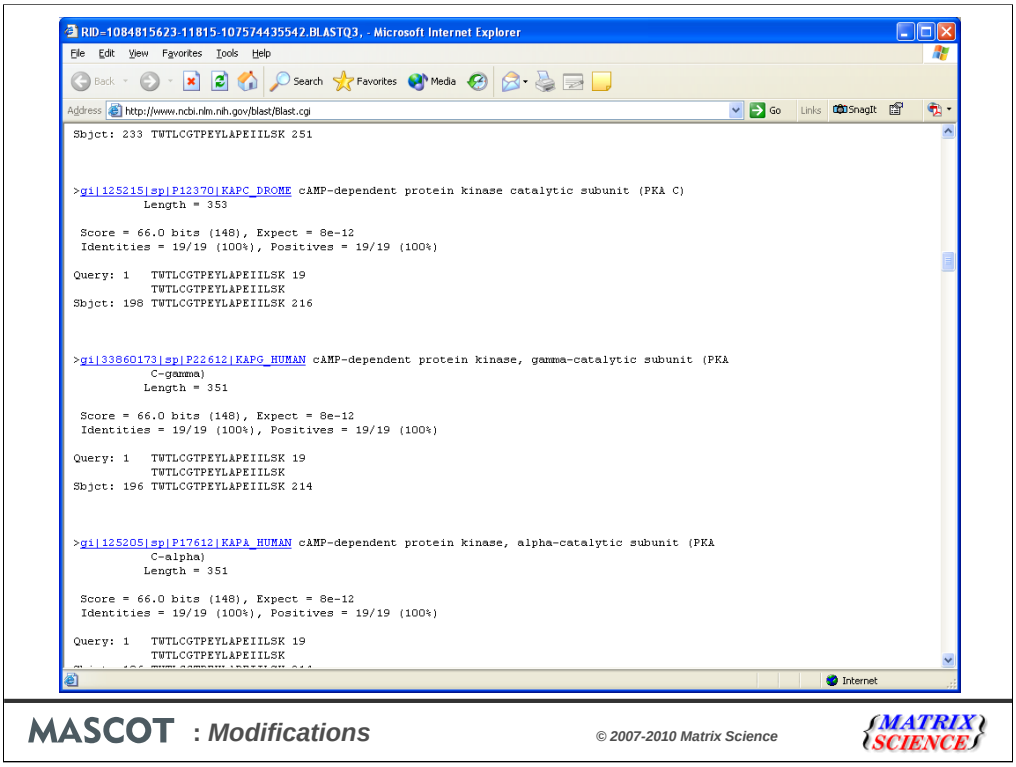

When there is ambiguity like this, the smart thing to do is to look for additional evidence. One option is to follow the link at the bottom of the peptide view report and run a Blast search of this peptide



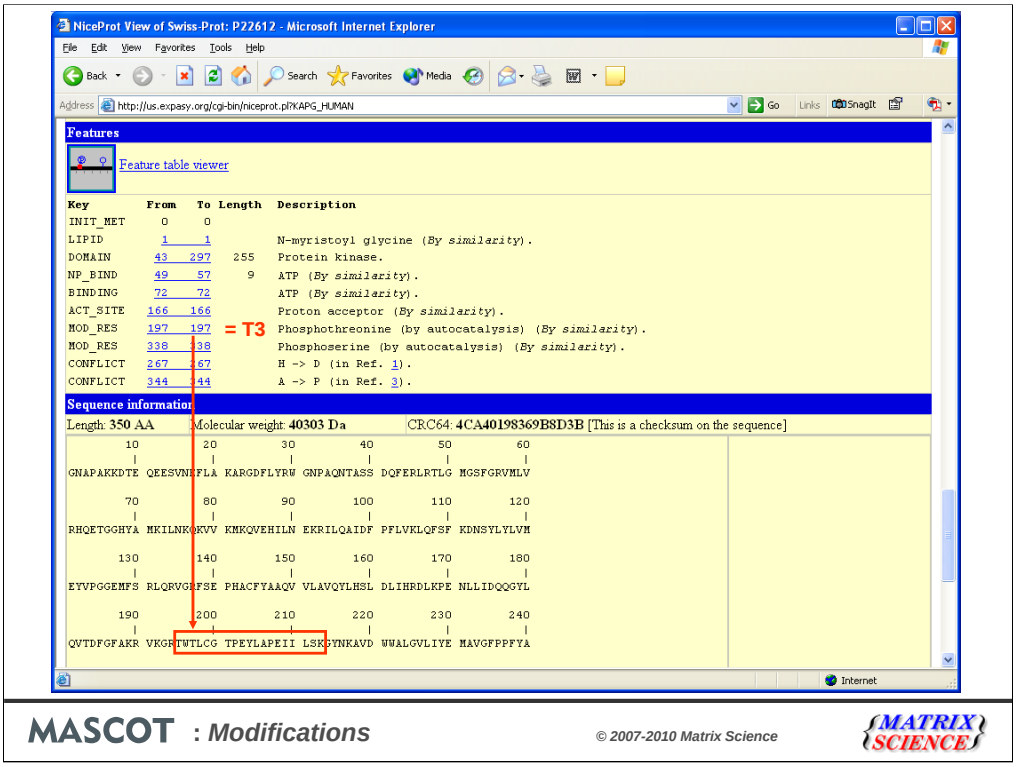





Choose Swiss-Prot as the database, because we want a database with good annotations




Lots of identity matches to this very common protein. Choose the relevant species, in this case human.

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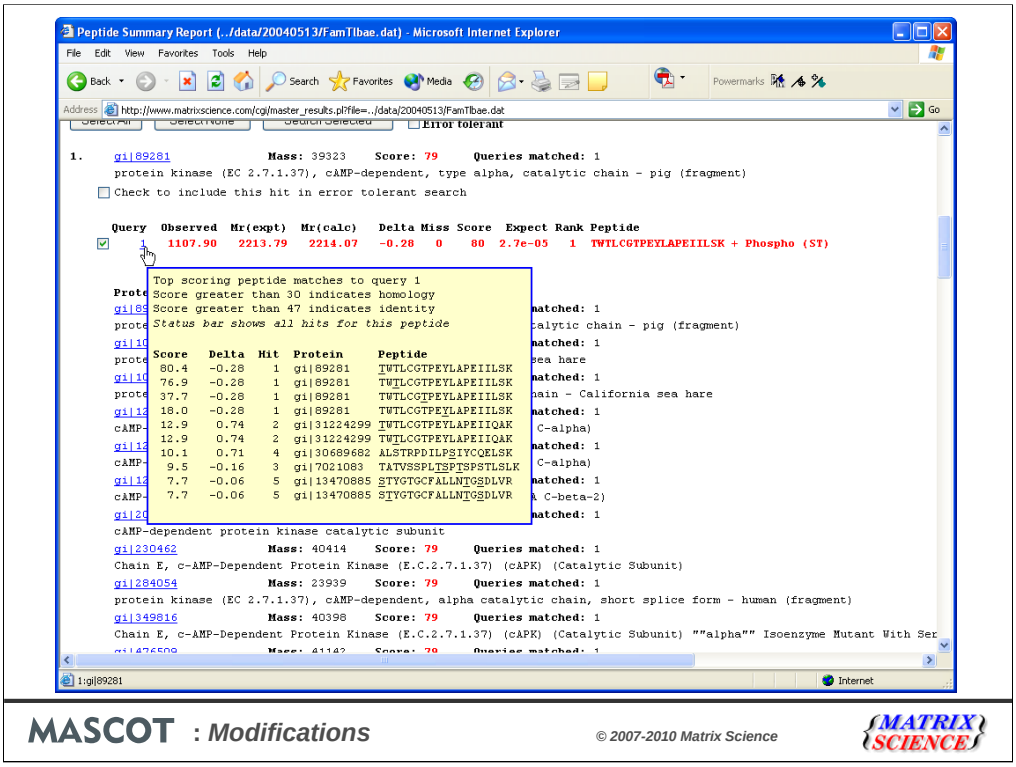
And hop over to Expasy to see the full text for this entry. Here is the peptide.

According to Swissprot, the phosphate is on T3, not T1

So, either Swiss-Prot is wrong or the extra match in the b series is spurious. I've no idea which. But, this does illustrate how easy it is to over-interpret noisy MS data.




The Mascot score reminds us that there is little to choose between T1 and T3. All we can say with confidence is that the phosphate is on one or the other … or maybe there is a mixture.

## Site Analysis

- If alternative sites differ by 20 in score, safe-ish to disregard lower one(s)
- If alternative sites have similar scores, you may be able to choose a preferred site by inspection
- Often, you just can't differentiate between closely spaced sites, even with great data.



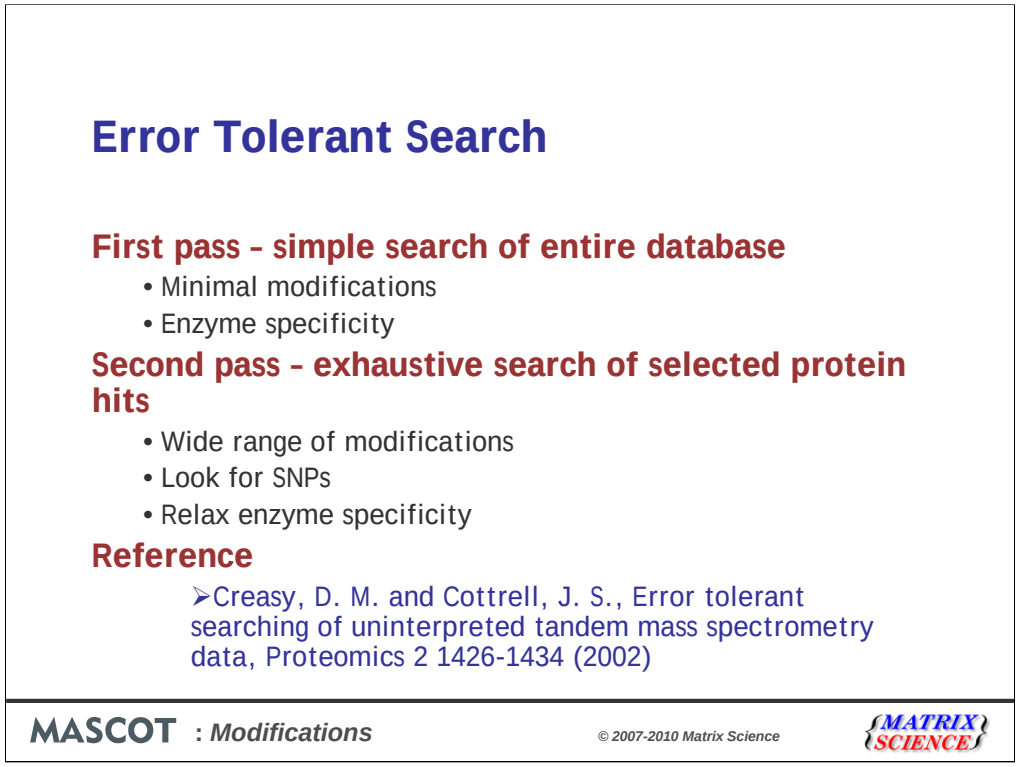


These are our suggested guidelines when using Mascot for site analysis:

If alternative sites differ by 20 in score, safe-ish to disregard lower one(s)

If alternative sites have similar scores, you may be able to choose one by inspection. But, be careful … one peak is just one peak

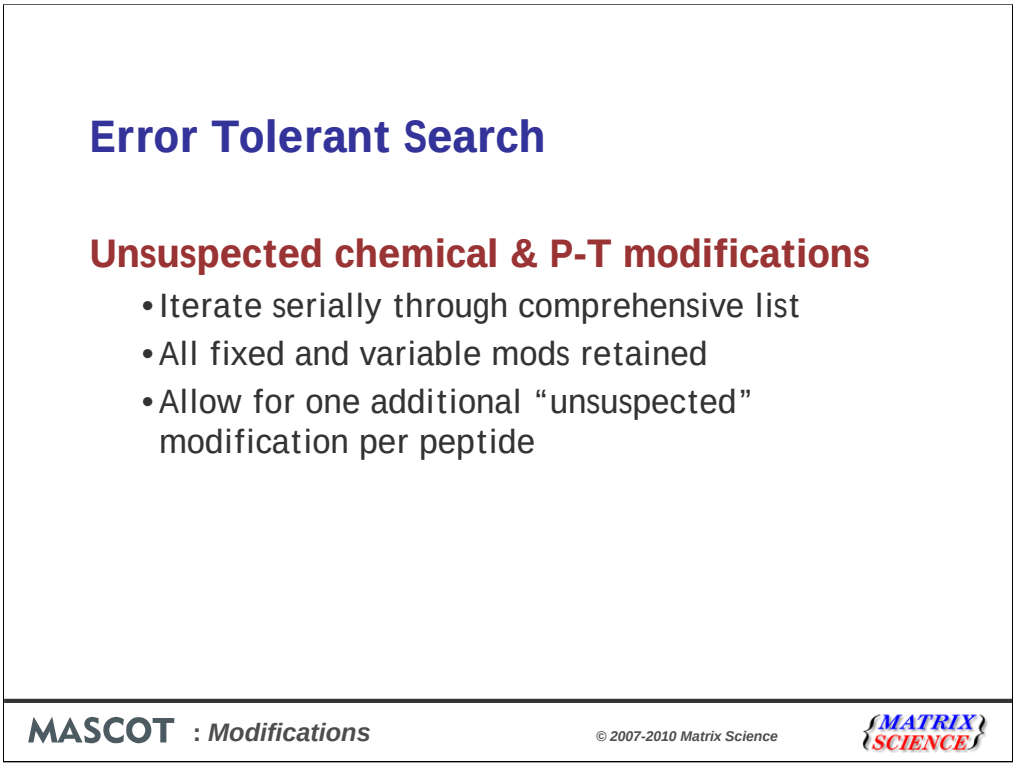
Often, you just can't differentiate between adjacent sites, even with great data.




Now, back to the challenge of finding PT modifications. There are many hundreds of modifications in Unimod, yet I've emphasised the importance of using the minimum number of variable modifications in a search. So, how are we supposed to find unusual modifications?

If you are searching uninterpreted MS/MS data, the efficient way to find unusual modifications, as well as variations in the primary sequence, is a two pass search. The first pass search is a simple search of the entire database with minimal modifications. The protein hits found in the first pass search are then selected for an exhaustive second pass search. During this second pass search, we can look for all possible modifications, sequence variants, and non-specific cleavage products.

Because only a handful of entries are being searched, search time is not an issue. The down side is that it is difficult to apply any kind of threshold to the results, or calculate expectation values, because the entries being searched have been pre-selected.




For modifications, an error tolerant search looks for one unsuspected modification per peptide in addition to those mods specified as fixed or variable. This is sufficient because it will be very rare to get two unsuspected mods on a single peptide

## Error Tolerant Search

### Primary sequence variants

- Protein database
  - Look for all single base substitutions
  - No attempt to find single base insertions & deletions because of frame shifts
- Nucleic acid database
  - Look for all single base substitutions, insertions & deletions





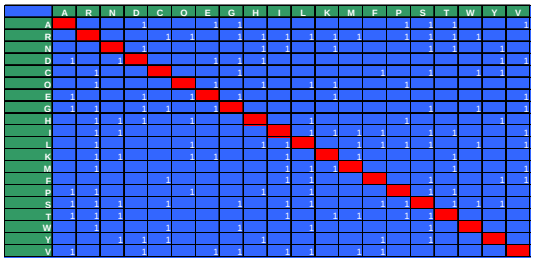
The error tolerant search also looks for sequence variants, such as single nucleotide polymorphisms (SNPs) or sequencing errors.

For a protein database, we can't look for the consequences of inserted or deleted bases, because these give rise to frame shifts, and the entire sequence changes from that point on.

## Error Tolerant Search

### Single base substitutions:

1. Back translate AA to codons
2. Perform all possible single base substitutions
3. Translate resulting codons to AA.



MASCOT : *Modifications*



MATRIX SCIENCE

With a protein database, we don't just look for all possible residue substitutions. Many of these simply don't happen in nature. The observed substitutions are those that correspond to base substitutions in the DNA.

## Error Tolerant Search

**The following constraints apply to the standard, first pass search:**

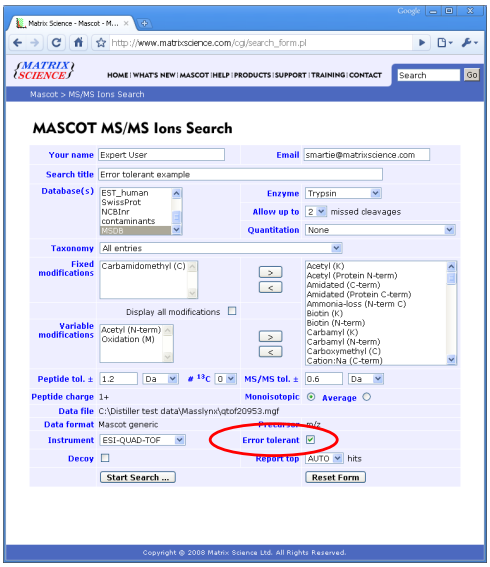
1. Enzyme must be fully specific
2. A reduced ceiling on the number of variable modifications, (default is 2, but this can be changed globally in mascot.dat or for a user group in Mascot security)
3. Cannot be combined with an automatic decoy database search
4. Cannot be combined with quantitation
5. Search cannot include error tolerant sequence tag





There are some constraints on the standard, first pass search

## Error Tolerant Search

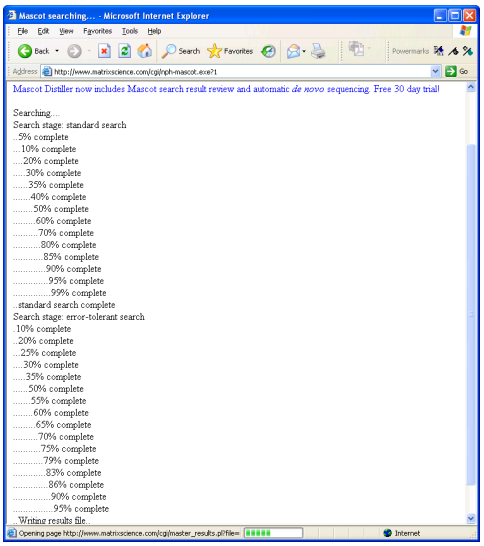






Otherwise, submitting the search is just like submitting a standard search except that you check the Error Tolerant Checkbox

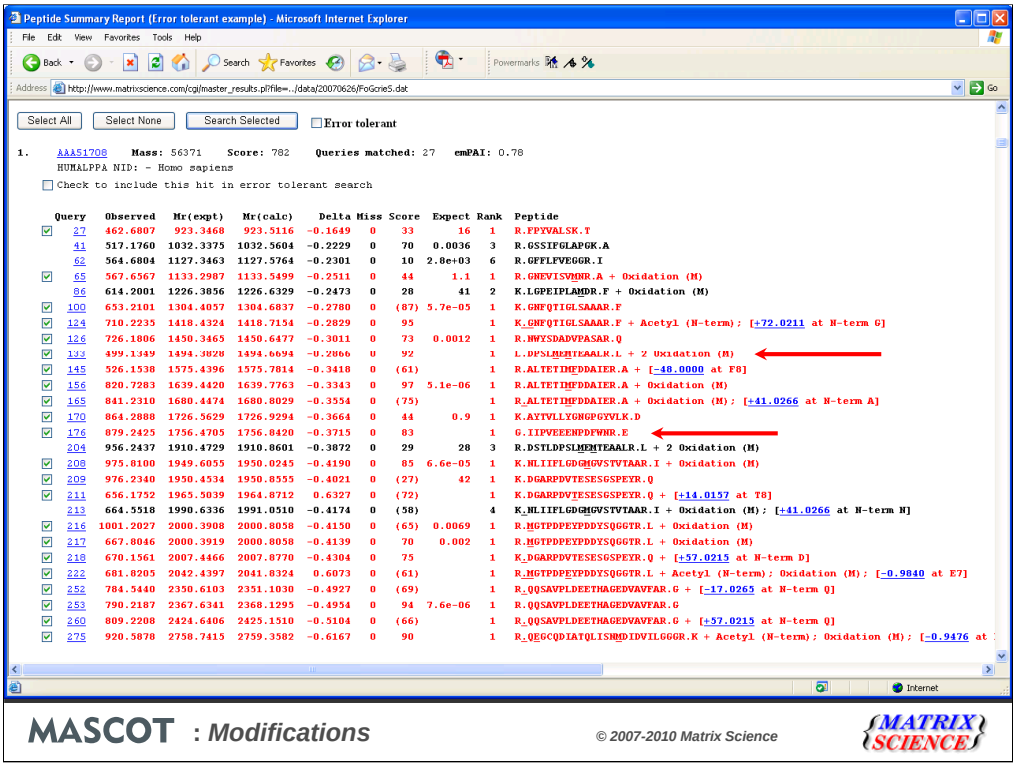
## Error Tolerant Search








You see two sets of progress reports




And here is the first hit of the results report. The additional matches, found in the error tolerant search, are the ones without Expect values. This is because they have been obtained by selecting a small number of database entries and beating them into submission with non-specificity, substitutions and a long list of modifications. This makes it difficult to apply any meaningful measure of statistical significance.

One of these, query 133, is a simple, non-specific peptide with a very good score. There's another example for query 176. The error tolerant search is a much better way of picking up non-specific peptides than searching the entire database with semi-trypsin or no enzyme. We only fail to get such matches in an error tolerant search if there are no matches to the protein in the first pass search. However, you have to ask yourself whether you would believe a protein hit in which the only peptide match was non-specific. I think the answer is no.

## Error Tolerant Search

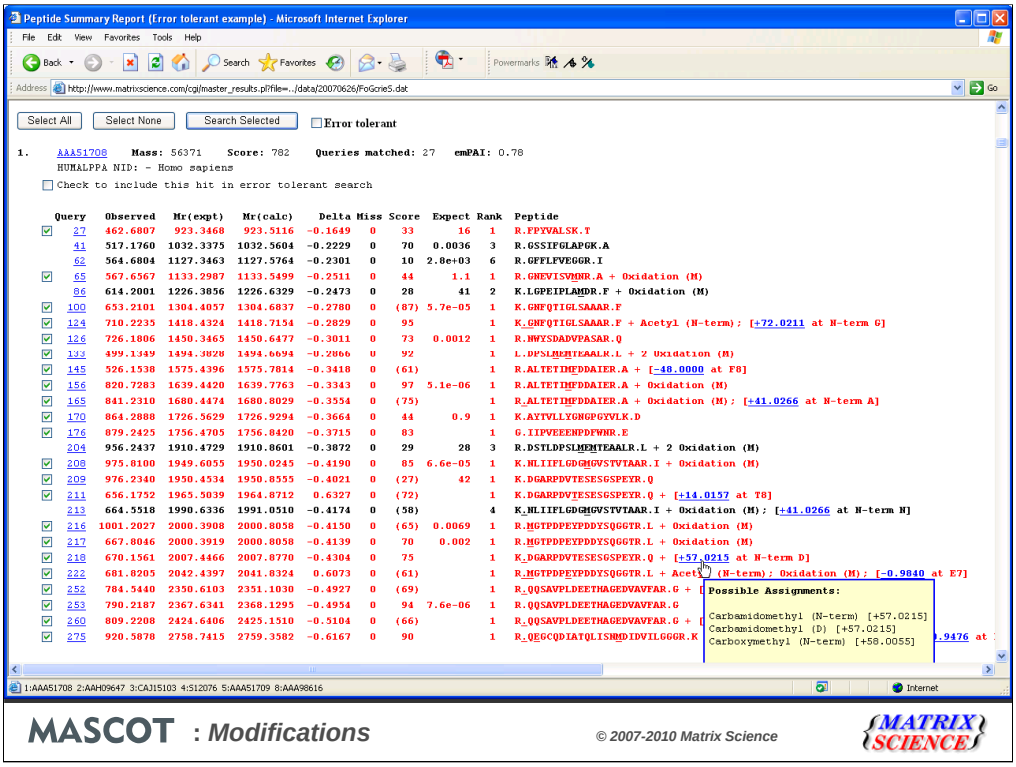
### To reduce 'junk' matches

- An individual peptide can be semi-specific OR have one unsuspected modification OR have one primary sequence mutation.
- If the mass delta of the modification is less than the smaller of the precursor mass tolerance and the fragment mass tolerance, the modification is rejected. This eliminates modifications that are meaningless given the estimated mass error, like Q->K, in most cases.
- Match must have a score of at least the identity threshold for the same query in the first pass search
- Match must have a score in excess of the highest scoring match to the same query in the first pass search

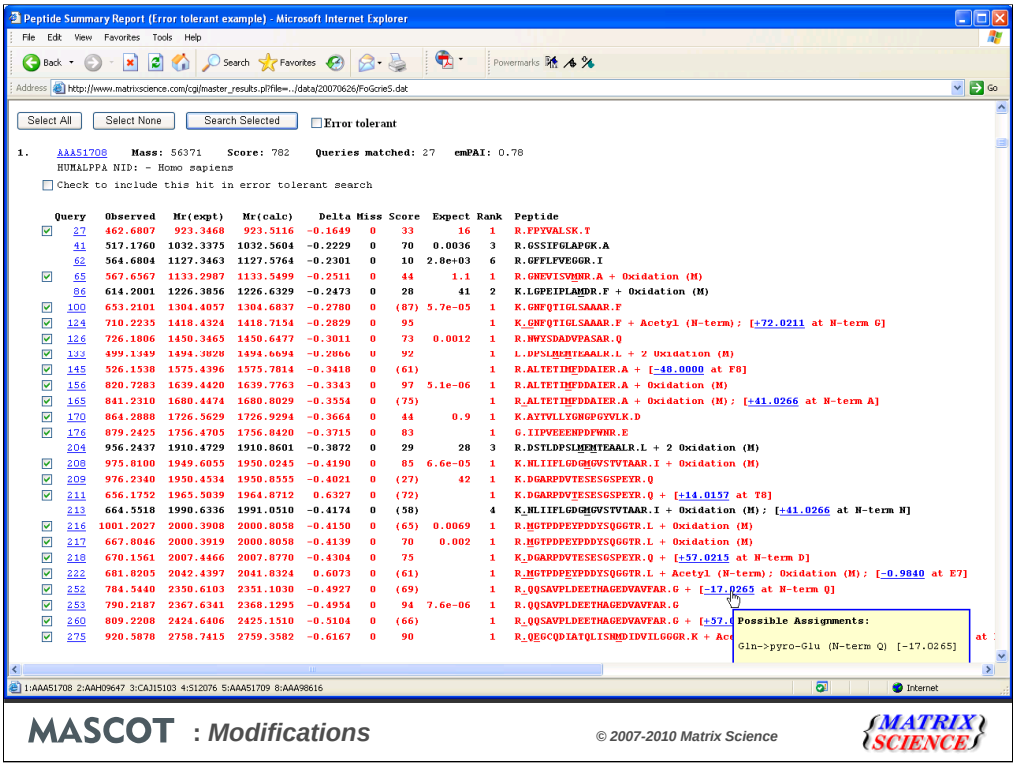




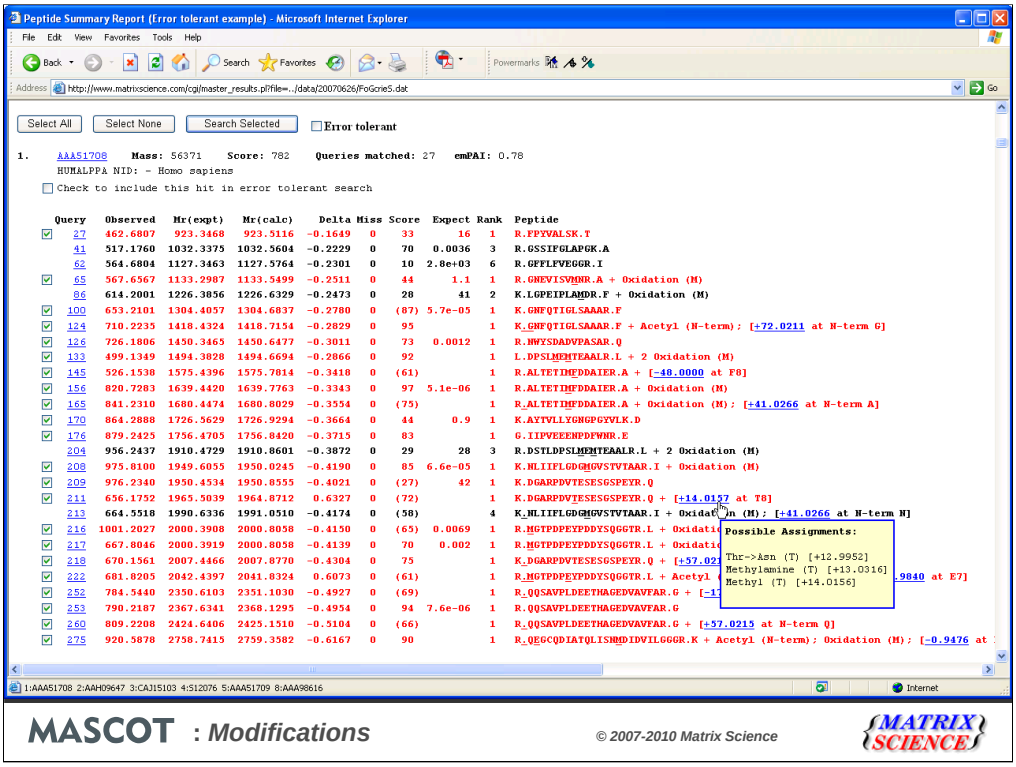
The matches from an error tolerant search are aggressively filtered to remove junk matches



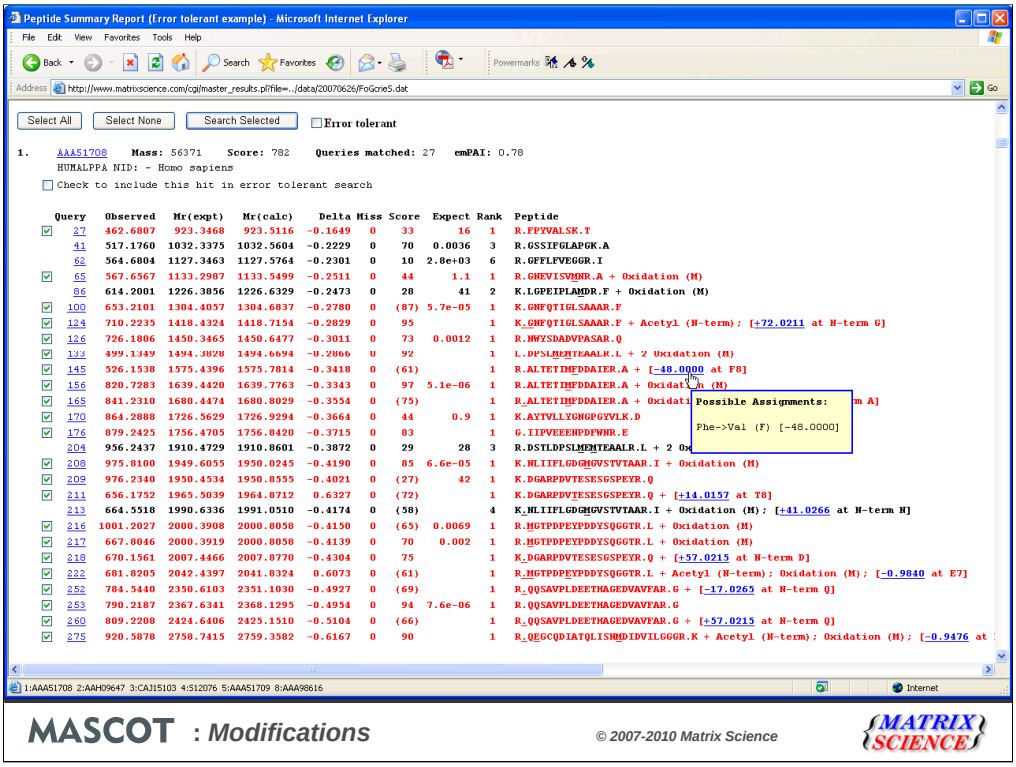

Take a look at the match to query 218. The mass tolerance for this search was fairly wide, so the observed mass difference could correspond to either carbamidomethylation or carboxymethylation at the N-terminus. Since this sample was alkylated with iodoacetamide, we would choose carbamidomethylation as the more likely suspect, especially as this brings the error on the precursor mass into line with the general trend, whereas carboxymethylation would give an error of +0.6 Da. The assignment to carbamidomethylation is also very believable, because this is a known artefact of over-alkylation. The same modification is found for query 260.




Another easily believable assignment is pyro-Glu for the match to query 252.

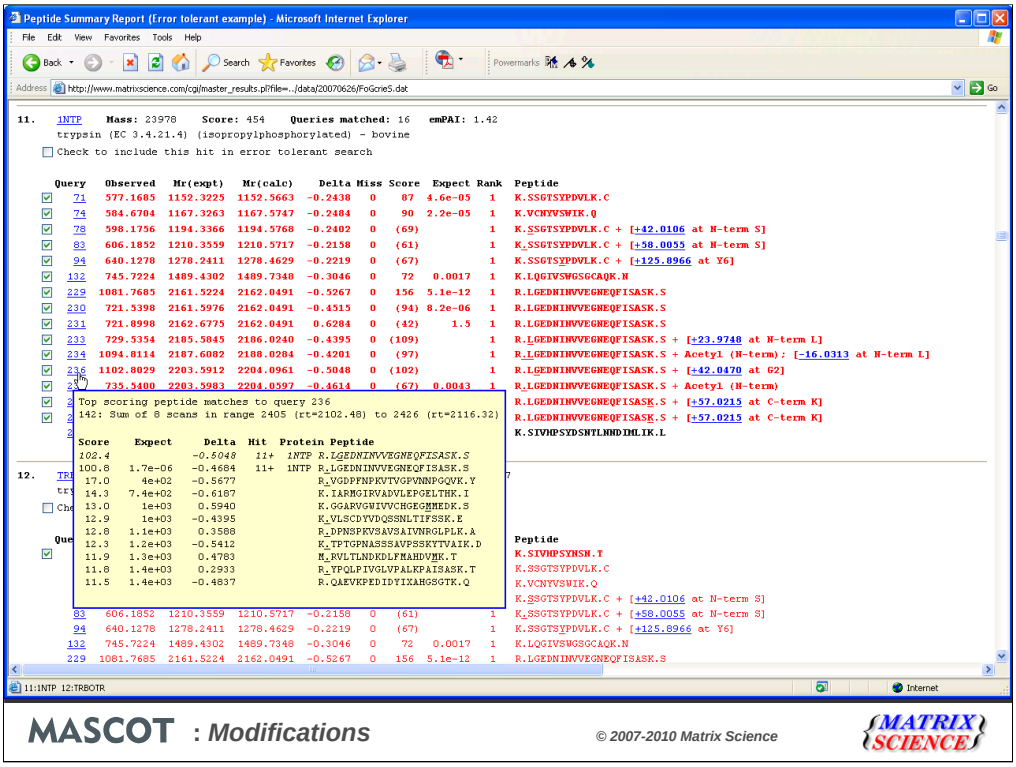



As is methylation ay T8 for query 211

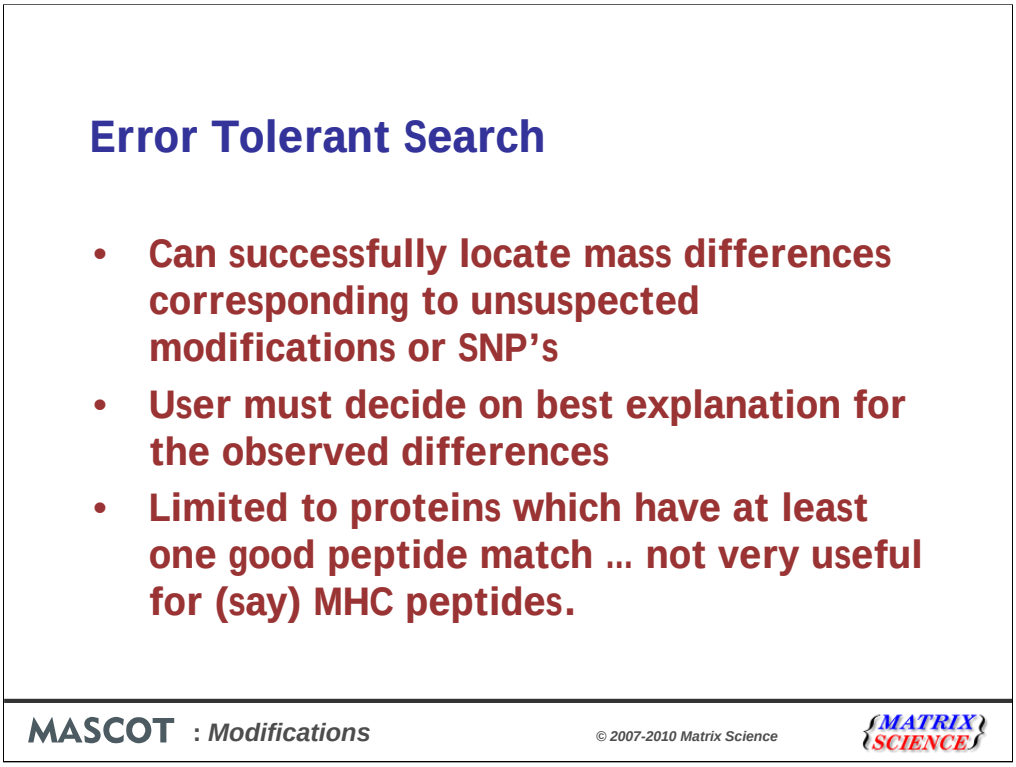



In other cases, the match may be good, but the assignment is not believable. Query 145 is listed with a substitution at F8 causing a loss of 48 Da. This seems unlikely because we have 2 other matches to the same peptide without any substitution. What else could it be? Well, notice that the other two matches are both oxidised at M7. If we suppose this peptide is also oxidised, then the mass shift becomes -64, which is a well-known loss for oxidised methionine, (loss of methanesulfenic acid). This would seem a much more likely explanation for this match.

It is important to understand that the error tolerant search finds new matches by introducing mass shifts at different positions in the database sequences. The match may be very strong, but figuring out a credible assignment can require a bit of detective work.




You should also look at the other yellow pop-up when trying to decide whether to accept a match or not. In this example, the error tolerant search was able to get a slightly higher score by shifting a modification of +42 Da from the amino terminus to the adjacent glycine. However, as score increase of 2 in 100 is negligible. Much more believeable to take the original match from the first pass search, which can be explained as N-terminal acetylation.




In summary, an error tolerant search

•Can successfully locate mass differences corresponding to unsuspected modifications or SNP's

•User must decide on best explanation for the observed differences

•Limited to proteins which have at least one good peptide match … not very useful for (say) MHC peptides