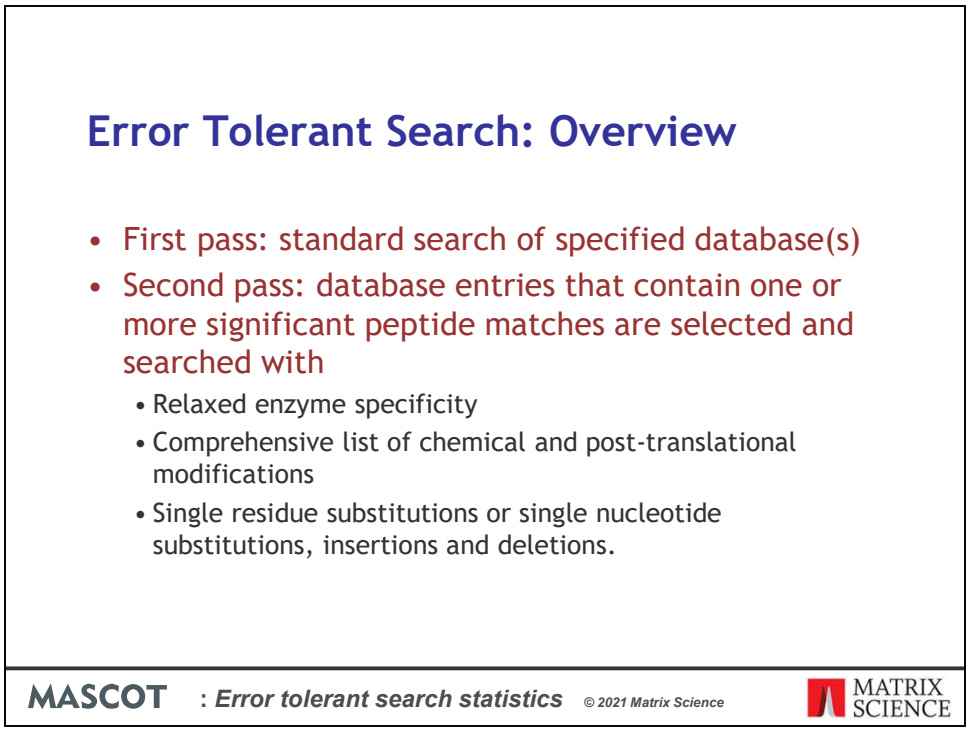
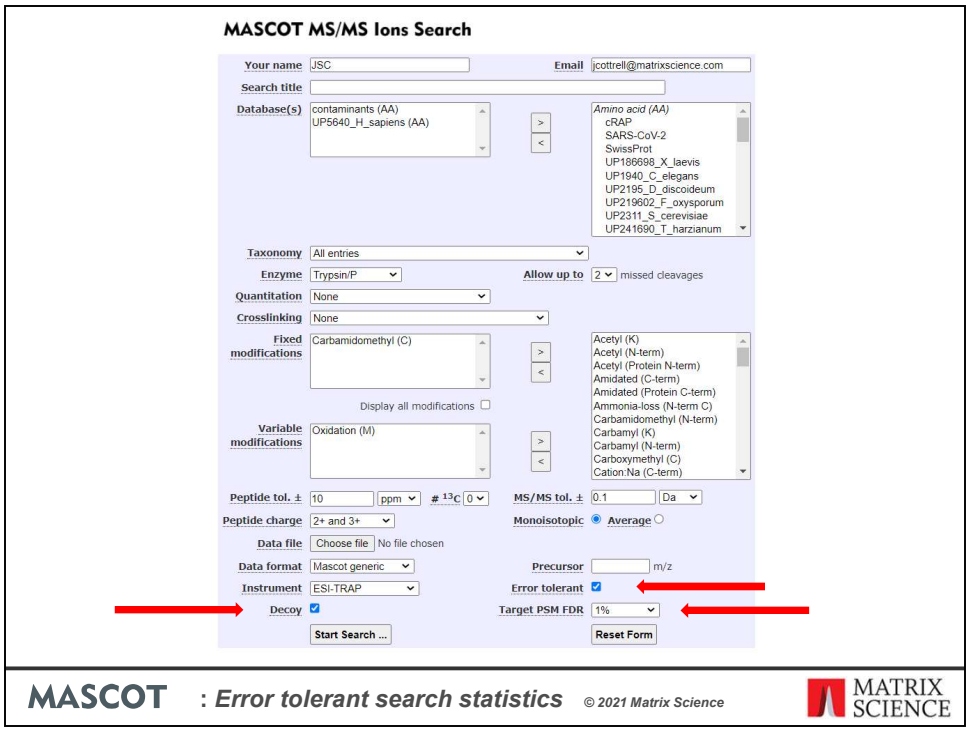
# Statistical Significance in Error Tolerant Search Results

I'd like to talk about some improvements we've made in the reporting of Error Tolerant search results




An error tolerant search is the most efficient way to find unsuspected modifications, non-specific cleavage products, and sequence variants.

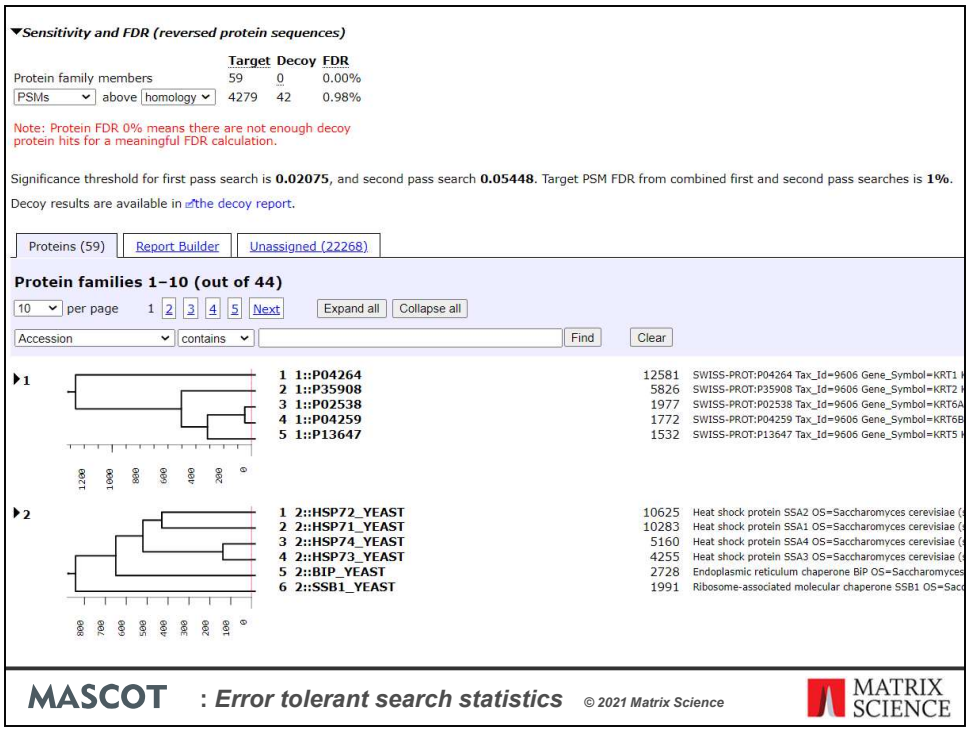



The big difference in Mascot Server 2.8 is that we now use target-decoy to assign significance to the all matches, including those found in the second pass search.

As in previous releases, all you need to do to perform an error tolerant search is to check a box on the search form.

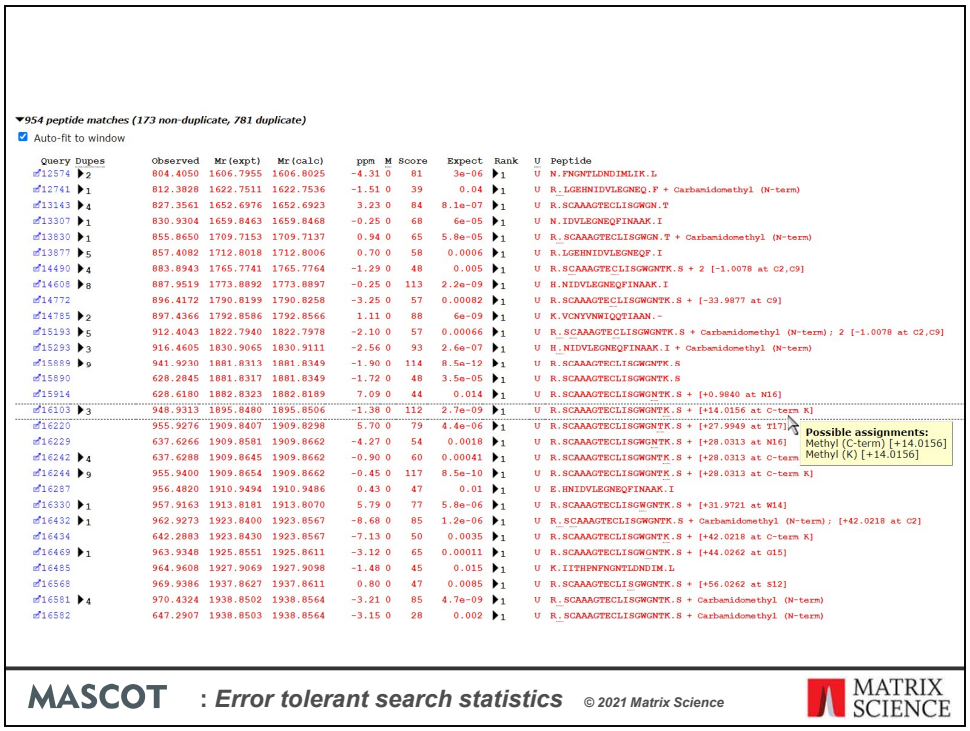
In Mascot Server 2.8, you can (and should) also check the box to use target-decoy. Without a decoy, expect values are derived from counting trials – that is, the number of candidate peptides that have been tested. This estimate is not always accurate; particularly when there is something wrong with the choice of database or search parameters, making a large fraction of potential matches unavailable. Ticking the checkbox to search a decoy database gives a solid, empirical basis for the statistics.

There is also a control to specify the required false discovery rate. The reason we ask for is up front is that the FDR determines the set of proteins selected for the second pass search. For example, the first pass search might identify significant peptide matches to 500 proteins at an FDR of 5%, and these are sent through to the second pass. If the FDR was reduced to 1%, the number of proteins selected for the second pass might drop to 400. Although the FDR can be tweaked at the report stage, this will not give perfectly identical results to setting the required FDR in the search form.




When the results come back, you have a single report that combines the results from both passes.

The required FDR is applied independently to the results from the first and second pass searches. Since this is based on counts of PSMs, it also holds true for the combined results.



▼954 peptide matches (173 non-duplicate, 781 duplicate)

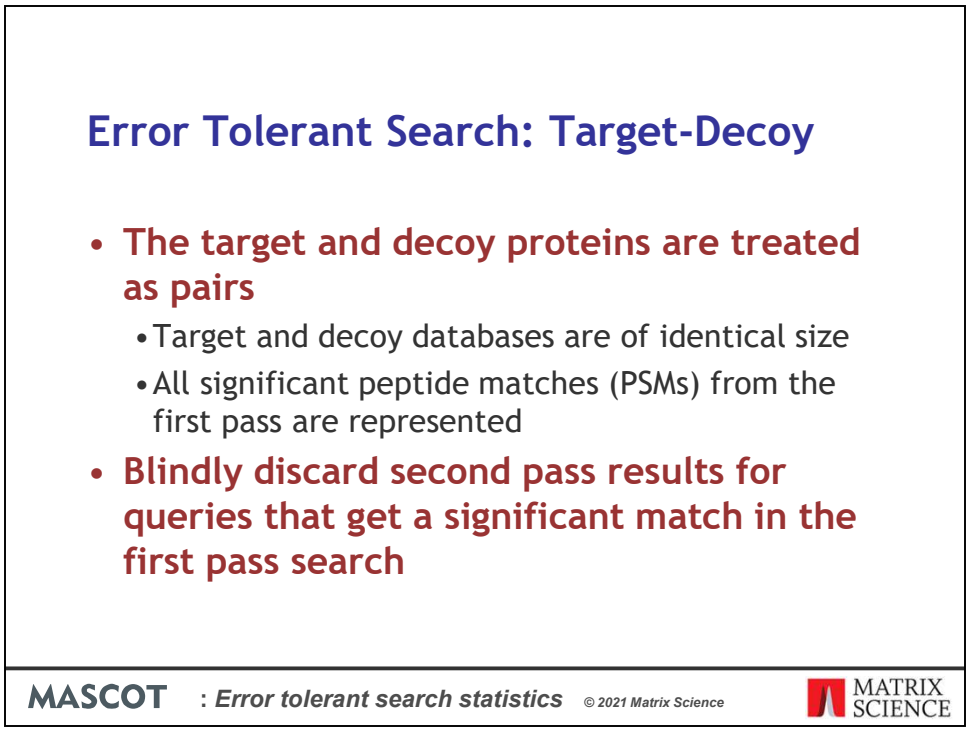
☑ Auto-fit to window

| Query | Dupes | Observed | Mr(expt) | Mr(calc) | ppm | M | Score | Expect | Rank | U | Peptide |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 12574 | ▶2 | 804.4050 | 1606.7955 | 1606.8025 | -4.31 | 0 | 81 | 3e-06 | ▶1 | U | N.FNGNTLDNDIMLIK.L |
| 12741 | ▶1 | 812.3828 | 1622.7511 | 1622.7536 | -1.51 | 0 | 39 | 0.04 | ▶1 | U | R.LGEHNIDVLEGNEQ.F + Carbamidomethyl (N-term) |
| 13143 | ▶4 | 827.3561 | 1652.6976 | 1652.6923 | 3.23 | 0 | 84 | 8.1e-07 | ▶1 | U | R.SCAAAGTECLISGWGN.T |
| 13307 | ▶1 | 830.9304 | 1659.8463 | 1659.8468 | -0.25 | 0 | 68 | 6e-05 | ▶1 | U | N.IDVLEGNEQFINAAK.I |
| 13830 | ▶1 | 855.8650 | 1709.7153 | 1709.7137 | 0.94 | 0 | 65 | 5.8e-05 | ▶1 | U | R.SCAAAGTECLISGWGN.T + Carbamidomethyl (N-term) |
| 13877 | ▶5 | 857.4082 | 1712.8018 | 1712.8006 | 0.70 | 0 | 58 | 0.0006 | ▶1 | U | R.LGEHNIDVLEGNEQF.I |
| 14490 | ▶4 | 883.8943 | 1765.7741 | 1765.7764 | -1.29 | 0 | 48 | 0.005 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + 2 [-1.0078 at C2,C9] |
| 14608 | ▶8 | 887.9519 | 1773.8892 | 1773.8897 | -0.25 | 0 | 113 | 2.2e-09 | ▶1 | U | H.NIDVLEGNEQFINAAK.I |
| 14772 | | 896.4172 | 1790.8199 | 1790.8258 | -3.25 | 0 | 57 | 0.00082 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [-33.9877 at C9] |
| 14785 | ▶2 | 897.4366 | 1792.8586 | 1792.8566 | 1.11 | 0 | 88 | 6e-09 | ▶1 | U | K.VCNYVNWIQQTIAAN.- |
| 15193 | ▶5 | 912.4043 | 1822.7940 | 1822.7978 | -2.10 | 0 | 57 | 0.00066 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + Carbamidomethyl (N-term); 2 [-1.0078 at C2,C9] |
| 15293 | ▶3 | 916.4605 | 1830.9065 | 1830.9111 | -2.56 | 0 | 93 | 2.6e-07 | ▶1 | U | H.NIDVLEGNEQFINAAK.I + Carbamidomethyl (N-term) |
| 15889 | ▶9 | 941.9230 | 1881.8313 | 1881.8349 | -1.90 | 0 | 114 | 8.5e-12 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S |
| 15890 | | 628.2845 | 1881.8317 | 1881.8349 | -1.72 | 0 | 48 | 3.5e-05 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S |
| 15914 | | 628.6180 | 1882.8323 | 1882.8189 | 7.09 | 0 | 44 | 0.014 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [+0.9840 at N16] |
| 16103 | ▶3 | 948.9313 | 1895.8480 | 1895.8506 | -1.38 | 0 | 112 | 2.7e-09 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [+14.0156 at C-term K] |
| 16220 | | 955.9276 | 1909.8407 | 1909.8298 | 5.70 | 0 | 79 | 4.4e-06 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [+27.9949 at T17] |
| 16229 | | 637.6266 | 1909.8581 | 1909.8662 | -4.27 | 0 | 54 | 0.0018 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [+28.0313 at N16] |
| 16242 | ▶4 | 637.6288 | 1909.8645 | 1909.8662 | -0.90 | 0 | 60 | 0.00041 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [+28.0313 at C-term |
| 16244 | ▶9 | 955.9400 | 1909.8654 | 1909.8662 | -0.45 | 0 | 117 | 8.5e-10 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [+28.0313 at C-term K] |
| 16287 | | 956.4820 | 1910.9494 | 1910.9486 | 0.43 | 0 | 47 | 0.01 | ▶1 | U | E.HNIDVLEGNEQFINAAK.I |
| 16330 | ▶1 | 957.9163 | 1913.8181 | 1913.8070 | 5.79 | 0 | 77 | 5.8e-06 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [+31.9721 at W14] |
| 16432 | ▶1 | 962.9273 | 1923.8400 | 1923.8567 | -8.68 | 0 | 85 | 1.2e-06 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + Carbamidomethyl (N-term); [+42.0218 at C2] |
| 16434 | | 642.2883 | 1923.8430 | 1923.8567 | -7.13 | 0 | 50 | 0.0035 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [+42.0218 at C-term K] |
| 16469 | ▶1 | 963.9348 | 1925.8551 | 1925.8611 | -3.12 | 0 | 65 | 0.00011 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [+44.0262 at G15] |
| 16485 | | 964.9608 | 1927.9069 | 1927.9098 | -1.48 | 0 | 45 | 0.015 | ▶1 | U | K.IITHPNFNGNTLDNDIM.L |
| 16568 | | 969.9386 | 1937.8627 | 1937.8611 | 0.80 | 0 | 47 | 0.0085 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + [+56.0262 at S12] |
| 16581 | ▶4 | 970.4324 | 1938.8502 | 1938.8564 | -3.21 | 0 | 85 | 4.7e-09 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + Carbamidomethyl (N-term) |
| 16582 | | 647.2907 | 1938.8503 | 1938.8564 | -3.15 | 0 | 28 | 0.002 | ▶1 | U | R.SCAAAGTECLISGWGNTK.S + Carbamidomethyl (N-term) |

Possible assignments:
Methyl (C-term) [+14.0156]
Methyl (K) [+14.0156]

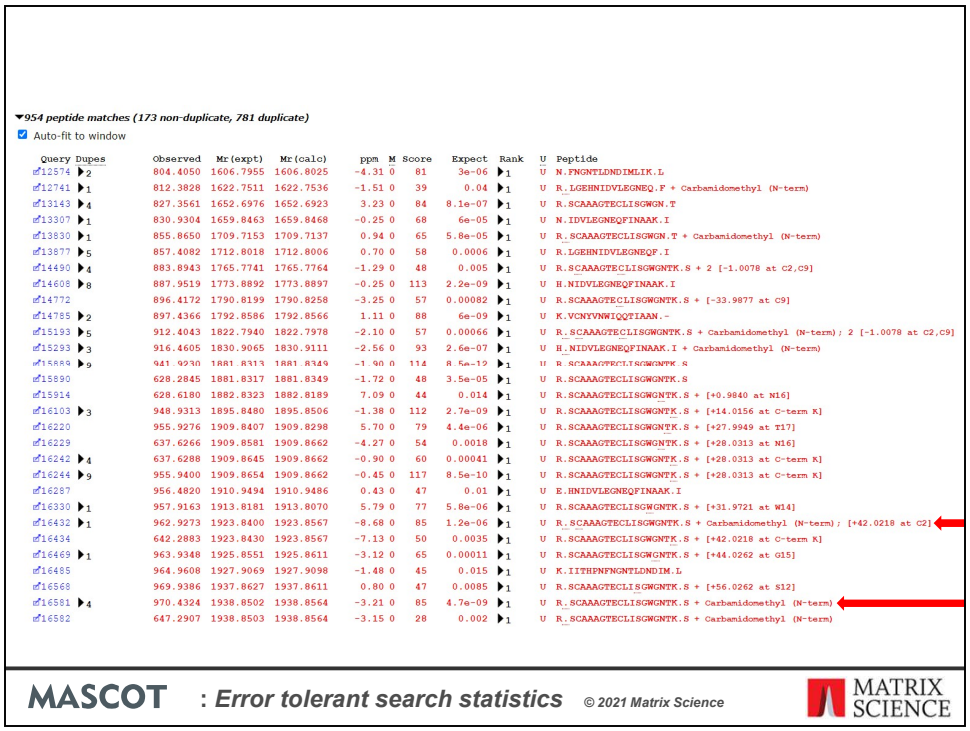
MASCOT : *Error tolerant search statistics* © 2021 Matrix Science MATRIX SCIENCE

Expect values are reported for both first and second pass matches. In earlier versions of Mascot, an error tolerant search could not be combined with target-decoy, and expect values based on counting trials were only reported for first pass matches.




The way it works is that target and decoy proteins are treated as pairs. After the first pass search, when proteins are selected, each significant match, whether target or decoy, causes the relevant pair of target and decoy proteins to be selected for the second pass. This means that the target and decoy databases are of identical size and contain all significant peptide matches (PSMs) from the first pass.

If a query gets a significant match in the first pass search, this is what we report, and we blindly discard the second pass results for this query. Sometimes, this means a stronger match is missed, but to do otherwise would be statistically dishonest. For example, if the significance threshold for a particular query in the first pass search corresponds to a score of 40, and we get a match with a score of 52, this is what we report, even if the second pass search might give us an even better match. This is not ideal, but the alternative is to burden all matches with statistics based on both passes. To illustrate why this is a problem, imagine we were to look at the second pass results and find nothing better. Now, we have a larger search space and the score threshold has increased to 55, so we have to discard our first pass match with a score of 52 because it is no longer significant.



954 peptide matches (173 non-duplicate, 781 duplicate)

Auto-fit to window

| Query | Dupes | Observed | Mr(expt) | Mr(calc) | ppm | M | Score | Expect | Rank | U | Peptide |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 12574 | 2 | 804.4050 | 1606.7955 | 1606.8025 | -4.31 | 0 | 81 | 3e-06 | 1 | U | N.FNGNTLDNDIMLIK.L |
| 12741 | 1 | 812.3828 | 1622.7511 | 1622.7536 | -1.51 | 0 | 39 | 0.04 | 1 | U | R.LGEHNIDVLEGNEQ.F + Carbamidomethyl (N-term) |
| 13143 | 4 | 827.3561 | 1652.6976 | 1652.6923 | 3.23 | 0 | 84 | 8.1e-07 | 1 | U | R.SCAAAGTECLISGWGN.T |
| 13307 | 1 | 830.9304 | 1659.8463 | 1659.8468 | -0.25 | 0 | 68 | 6e-05 | 1 | U | N.IDVLEGNEQFINAAK.I |
| 13830 | 1 | 855.8650 | 1709.7153 | 1709.7137 | 0.94 | 0 | 65 | 5.8e-05 | 1 | U | R.SCAAAGTECLISGWGN.T + Carbamidomethyl (N-term) |
| 13877 | 5 | 857.4082 | 1712.8018 | 1712.8006 | 0.70 | 0 | 58 | 0.0006 | 1 | U | R.LGEHNIDVLEGNEQF.I |
| 14490 | 4 | 883.8943 | 1765.7741 | 1765.7764 | -1.29 | 0 | 48 | 0.005 | 1 | U | R.SCAAAGTECLISGWGNTK.S + 2 [-1.0078 at C2,C9] |
| 14608 | 8 | 887.9519 | 1773.8892 | 1773.8897 | -0.25 | 0 | 113 | 2.2e-09 | 1 | U | H.NIDVLEGNEQFINAAK.I |
| 14772 | | 896.4172 | 1790.8199 | 1790.8258 | -3.25 | 0 | 57 | 0.00082 | 1 | U | R.SCAAAGTECLISGWGNTK.S + [-33.9877 at C9] |
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| 15293 | 3 | 916.4605 | 1830.9065 | 1830.9111 | -2.56 | 0 | 93 | 2.6e-07 | 1 | U | H.NIDVLEGNEQFINAAK.I + Carbamidomethyl (N-term) |
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| 16242 | 4 | 637.6288 | 1909.8645 | 1909.8662 | -0.90 | 0 | 60 | 0.00041 | 1 | U | R.SCAAAGTECLISGWGNTK.S + [+28.0313 at C-term K] |
| 16244 | 9 | 955.9400 | 1909.8654 | 1909.8662 | -0.45 | 0 | 117 | 8.5e-10 | 1 | U | R.SCAAAGTECLISGWGNTK.S + [+28.0313 at C-term K] |
| 16287 | | 956.4820 | 1910.9494 | 1910.9486 | 0.43 | 0 | 47 | 0.01 | 1 | U | E.HNIDVLEGNEQFINAAK.I |
| 16330 | 1 | 957.9163 | 1913.8181 | 1913.8070 | 5.79 | 0 | 77 | 5.8e-06 | 1 | U | R.SCAAAGTECLISGWGNTK.S + [+31.9721 at W14] |
| 16432 | 1 | 962.9273 | 1923.8400 | 1923.8567 | -8.68 | 0 | 85 | 1.2e-06 | 1 | U | R.SCAAAGTECLISGWGNTK.S + Carbamidomethyl (N-term); [+42.0218 at C2] |
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| 16485 | | 964.9608 | 1927.9069 | 1927.9098 | -1.48 | 0 | 45 | 0.015 | 1 | U | K.IITHPNFNGNTLDNDIM.L |
| 16568 | | 969.9386 | 1937.8627 | 1937.8611 | 0.80 | 0 | 47 | 0.0085 | 1 | U | R.SCAAAGTECLISGWGNTK.S + [+56.0262 at S12] |
| 16581 | 4 | 970.4324 | 1938.8502 | 1938.8564 | -3.21 | 0 | 85 | 4.7e-09 | 1 | U | R.SCAAAGTECLISGWGNTK.S + Carbamidomethyl (N-term) |
| 16582 | | 647.2907 | 1938.8503 | 1938.8564 | -3.15 | 0 | 28 | 0.002 | 1 | U | R.SCAAAGTECLISGWGNTK.S + Carbamidomethyl (N-term) |

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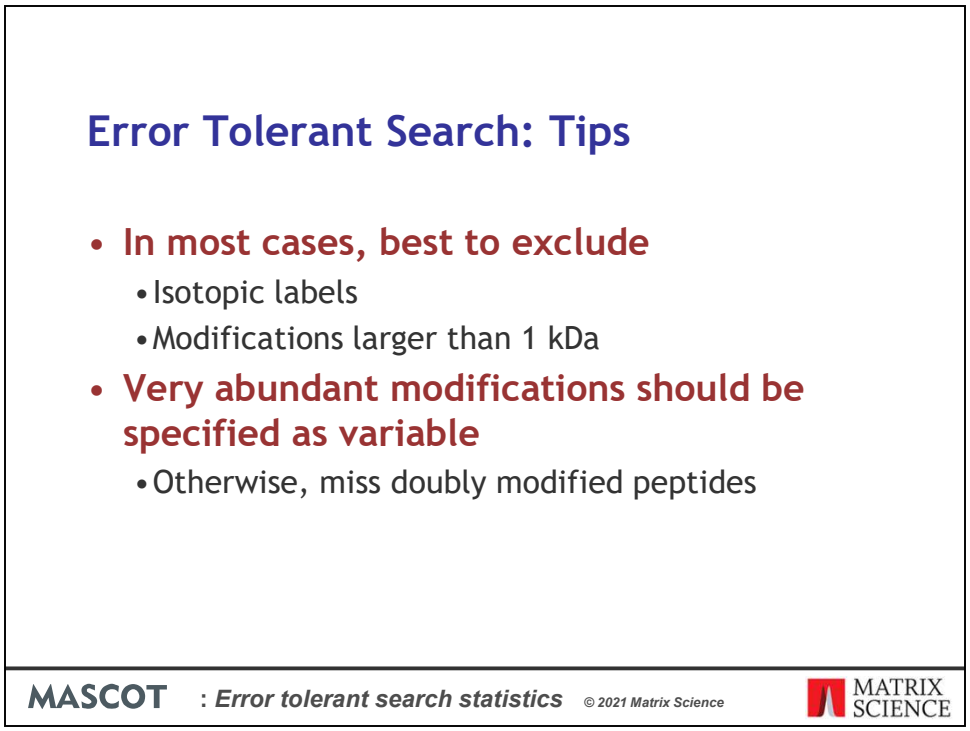
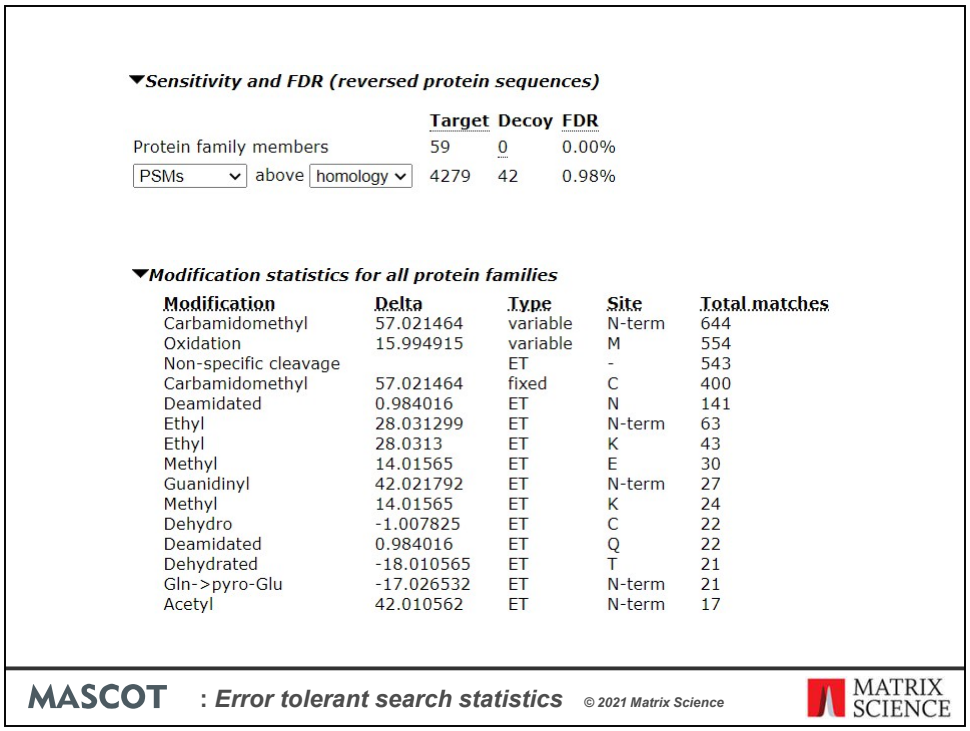
Usually, the search space for the second pass search is larger than for the first pass. This means that the significance threshold is more stringent for second pass matches. Here, for example, query 16581 gets a score of 85 in the first pass search which corresponds to an expect value of 4.7E-9. Query 16432 gets the same score in the second pass search, but the expect value is 1.2E-6, worse by a factor of 250




Finally, some tips.

The Unimod database contains a large number of entries that you do not expect to find in a general search. You can reduce the search time by excluding isotopic labels and very large modifications, which rarely give strong matches. 1 kDa is a good cut-off. Typically, this reduces the number of modifications by a third.

Remember that the second pass search works through the list of modifications serially. It doesn't look for combinations of modifications on a single peptide. So, if you have a very abundant modification, affecting 10% or more of the peptides, it is a good idea to specify this as a variable modification, so that you can find matches to peptides with both the abundant modification and an additional unsuspected one.



▼*Sensitivity and FDR (reversed protein sequences)*

| | Target | Decoy | FDR |
|---|---|---|---|
| Protein family members | 59 | 0 | 0.00% |
| PSMs above homology | 4279 | 42 | 0.98% |

▼*Modification statistics for all protein families*

| Modification | Delta | Type | Site | Total matches |
|---|---|---|---|---|
| Carbamidomethyl | 57.021464 | variable | N-term | 644 |
| Oxidation | 15.994915 | variable | M | 554 |
| Non-specific cleavage | | ET | - | 543 |
| Carbamidomethyl | 57.021464 | fixed | C | 400 |
| Deamidated | 0.984016 | ET | N | 141 |
| Ethyl | 28.031299 | ET | N-term | 63 |
| Ethyl | 28.0313 | ET | K | 43 |
| Methyl | 14.01565 | ET | E | 30 |
| Guanidinyl | 42.021792 | ET | N-term | 27 |
| Methyl | 14.01565 | ET | K | 24 |
| Dehydro | -1.007825 | ET | C | 22 |
| Deamidated | 0.984016 | ET | Q | 22 |
| Dehydrated | -18.010565 | ET | T | 21 |
| Gln->pyro-Glu | -17.026532 | ET | N-term | 21 |
| Acetyl | 42.010562 | ET | N-term | 17 |

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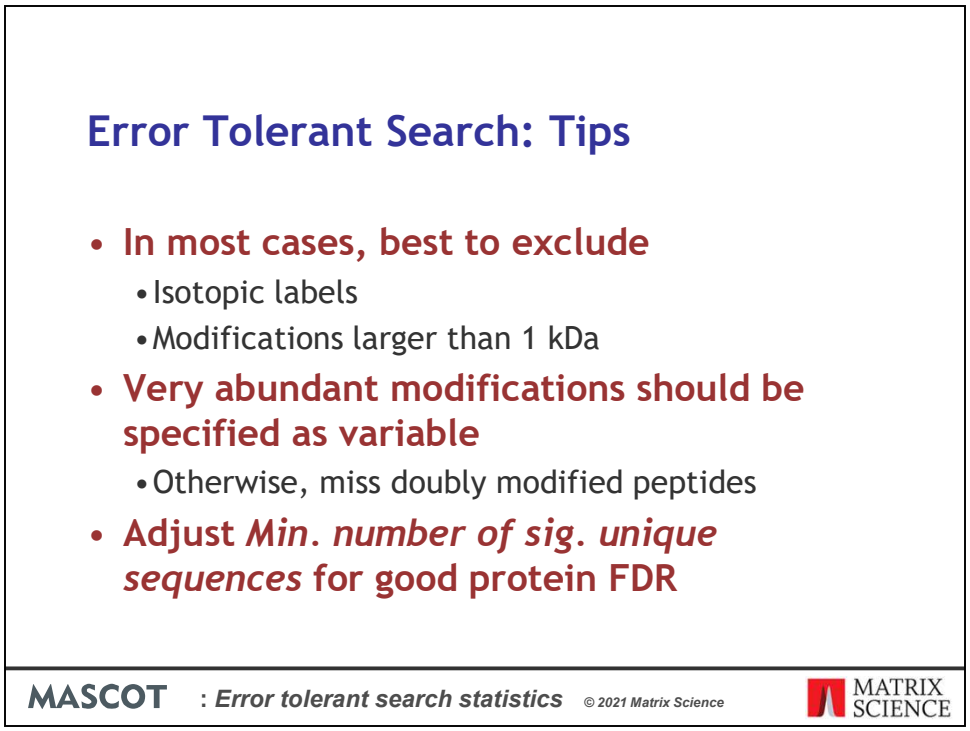
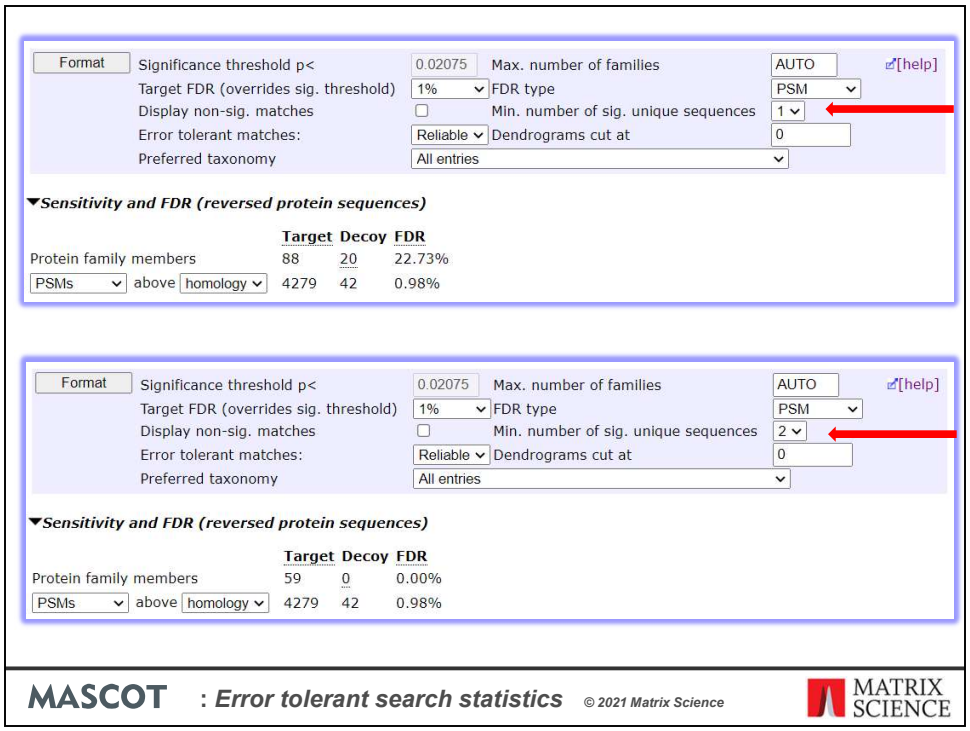
The example used for these screen shots was a search of LTQ-Orbitrap Velos data acquired by the Medical University of Graz and deposited in PRIDE project PXD002726. There is quite a bit of non-specific cleavage. N-term Carbamidomethyl was very common, so this was specified as a variable modification. The next most common modification is deamidation of asparagine, but it only affects 3% of the peptides, so it is not worth specifying as a variable modification.




Even when the FDR for PSMs is well controlled, the FDR for proteins will often be high for an error tolerant search because only a few entries are searched in the second pass.




In our example search, at 1% FDR for PSMs, the protein FDR is 23%, which sounds awful. This is simply because the 42 significant decoy matches are scattered randomly across 20 decoy proteins. If we increase the 'Min. number of sig. unique sequences' from 1 to 2 and choose 'Format', we eliminate one hit wonders, and the protein FDR drops to a more satisfactory 0%.