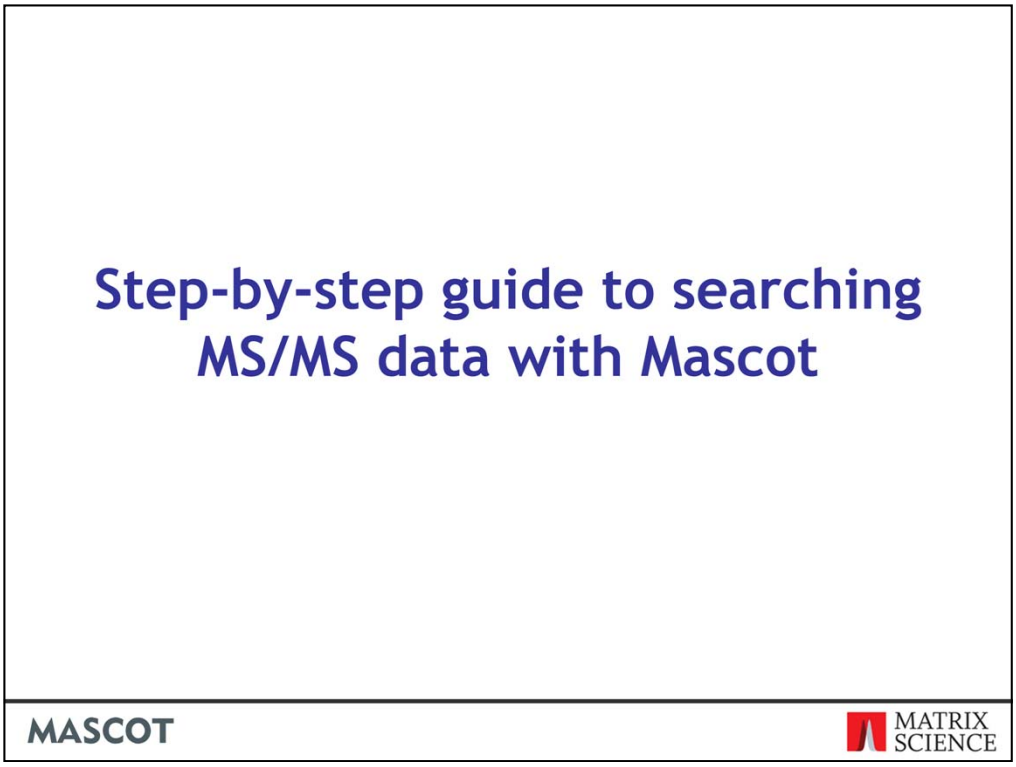
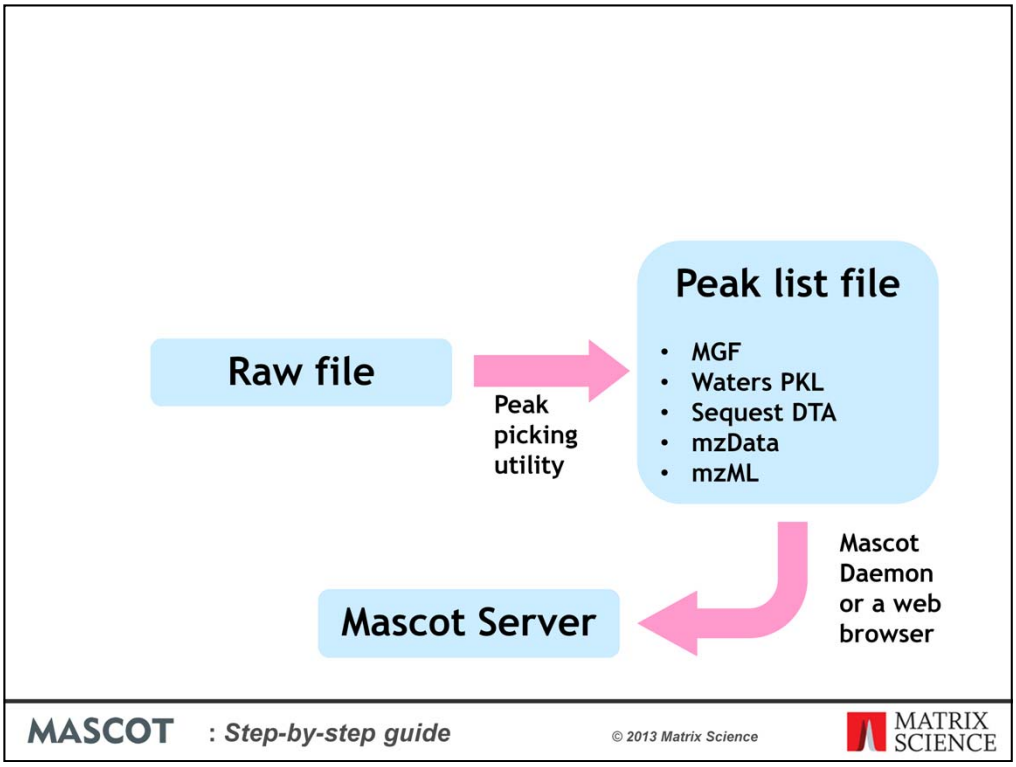
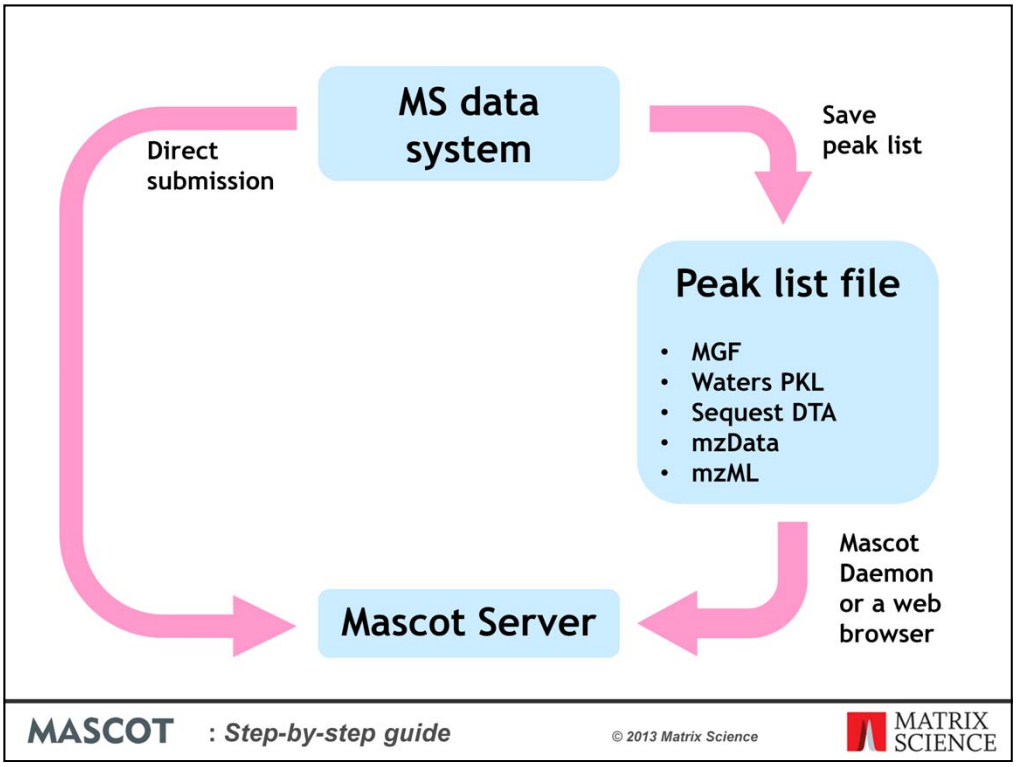


We are painfully aware that we don't have a good, introductory tutorial for Mascot on our web site. Its something that has come up in discussions many times, and we always resolve to do something but then get sidetracked. This talk is a dry run for a brief tutorial on searching MS/MS data. If you are new to database searching, I hope you will find it informative. If you are an experienced user, please give us your feedback on anything that is confusing or missing.

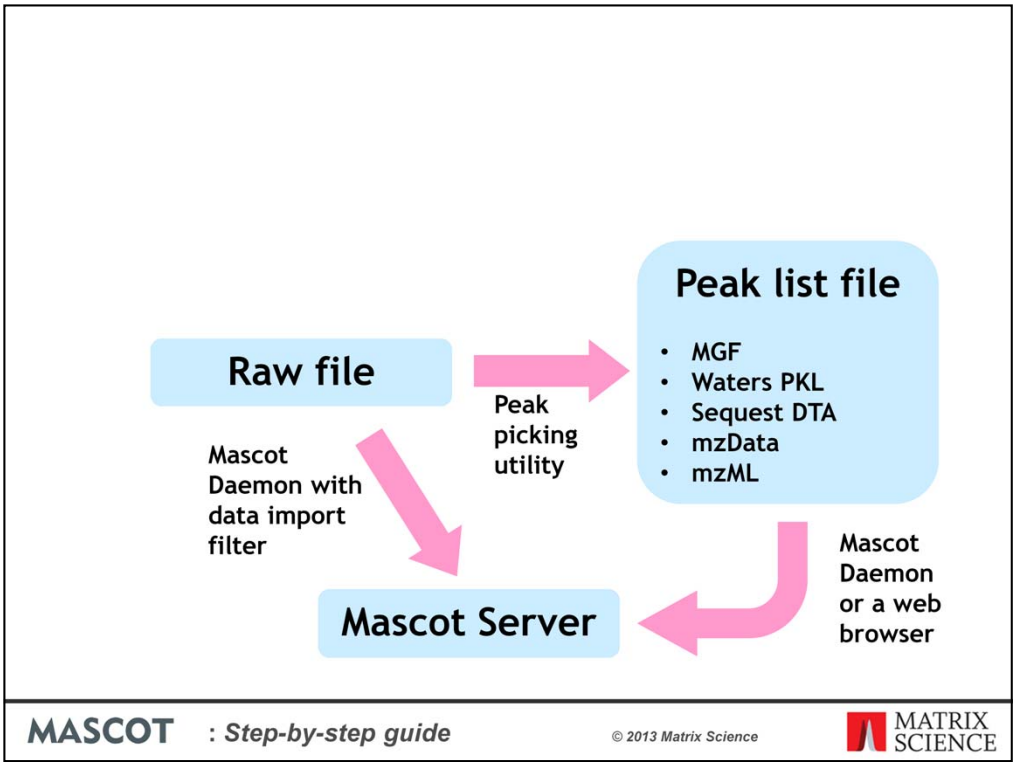


The first requirement for database searching with Mascot is a peak list; you cannot upload a raw data file. Raw data is converted into a peak list by a process called peak picking or peak detection.

Peak lists are text files and come in various different formats. If you have a choice, MGF is recommended, but you can also use any of the ones listed, plus a few others that are less widely used. Be careful with mzML, because this may contain either raw data or a peak list.

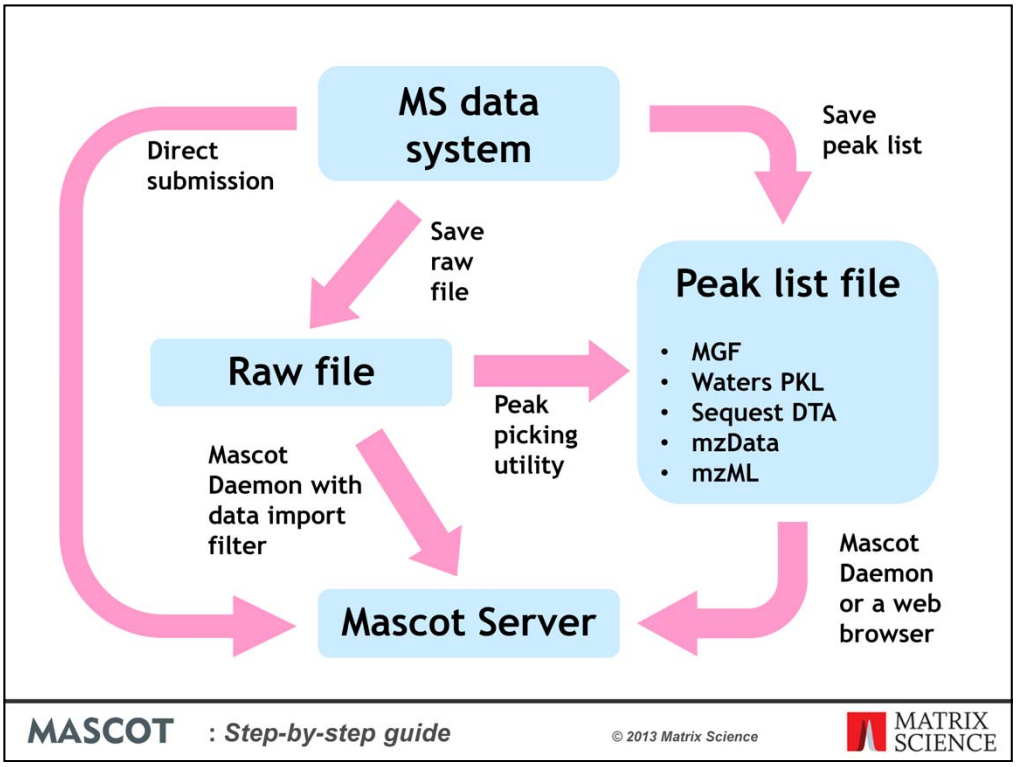


Often, the instrument data system takes care of peak picking, and you can submit a Mascot search directly from the data system or save a peak list to a disk file for submission using the web browser search form.

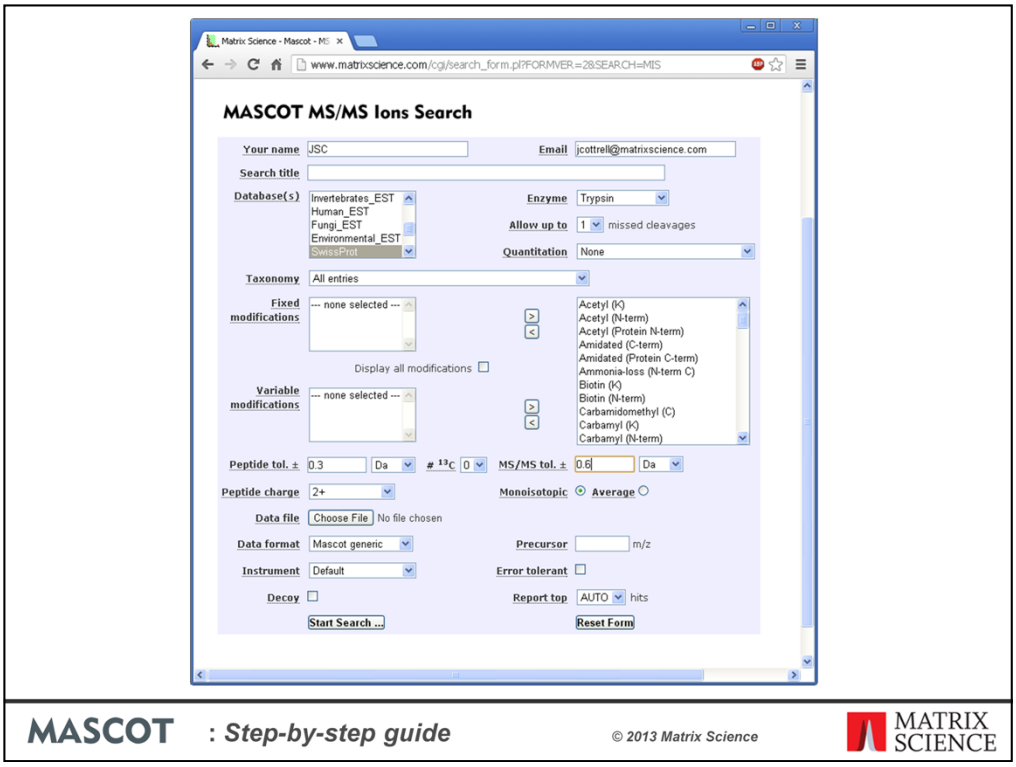


If not, or if you have a raw data file and no access to the data system, you'll need to find a utility to convert it into a peak list.

If you have Mascot in-house, you can also use Mascot Daemon to batch automate peak picking and search submission.



So, lots of ways to submit searches.



By itself, a peak list is not sufficient. There are also a number of search parameters that must be set appropriately. Here, we see the web browser search form for the current version of Mascot, 2.4. The labels for each control are also links to help topics.

The form looks much the same whether you have your own Mascot server, in-house, or whether you are connected to the free, public Mascot Server. If you are using the public Mascot Server, there are some restrictions, one of which is that you have to provide a name and email address so that we can email a link to your search results if the connection is broken. A more important restriction is that searches are limited to a maximum of 1200 spectra.

Whether you enter a search title is your choice. It is displayed at the top of the result report, and can be a useful way of identifying the search at some later time.

Golden Rule #1

- If at all possible, run a standard sample and use this to set all the search parameters.

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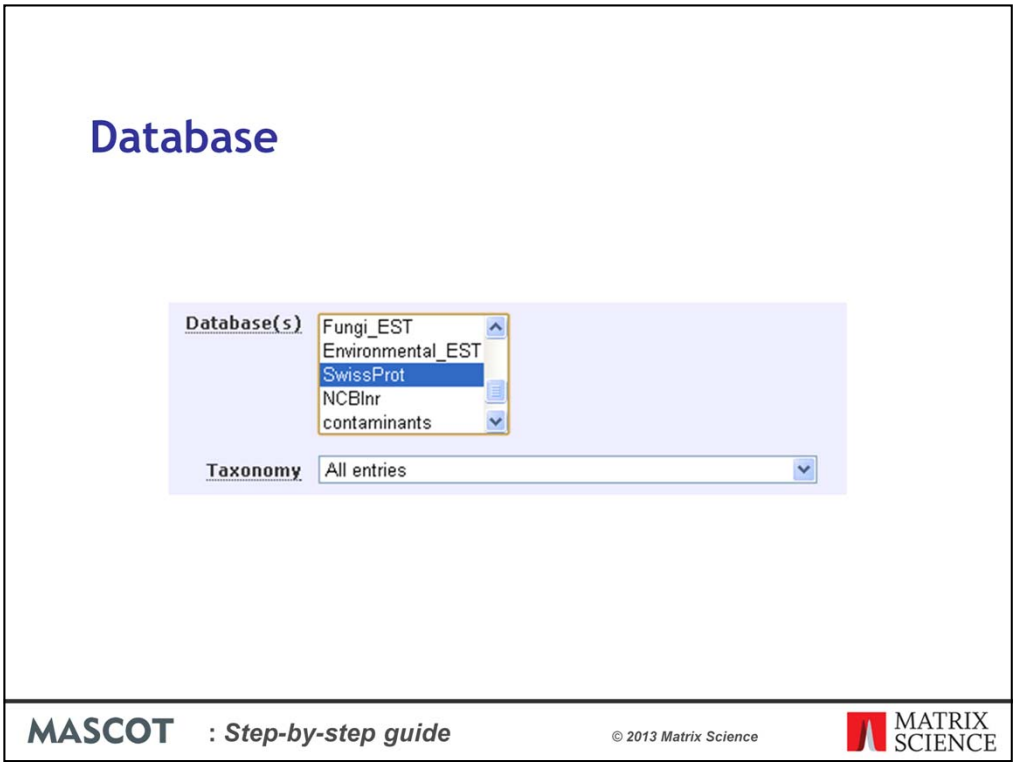
This brings us to our first 'golden rule'. If at all possible, run a standard sample and use this to set all the search parameters. By standard sample, I mean something like a BSA digest, which will give strong matches and where you know what the answer is supposed to be. Trying to set search parameters on an unknown is much more difficult, especially if the sample was lost somewhere during the work-up or if the instrument has developed a fault. I know this advice will often be ignored, but it is probably the single most important message of this talk.



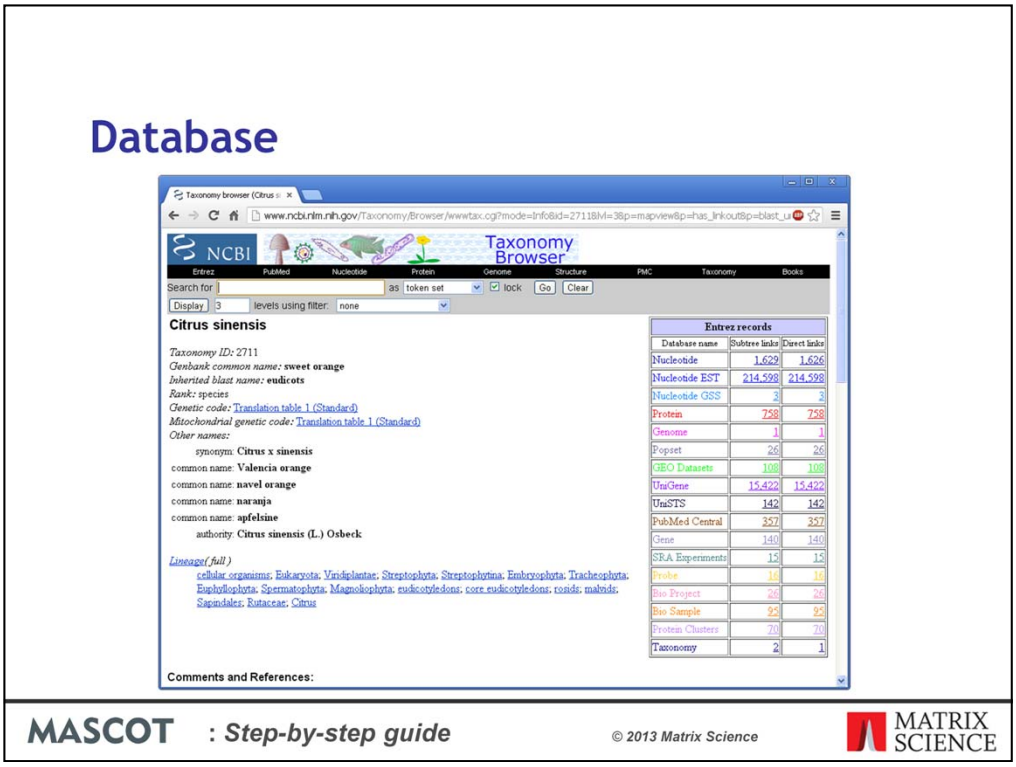
The first choice you have to make, and one of the more complicated, is which database to search. The free public Mascot Server has just a few of the more popular public databases, but an in-house server may have a hundred or more. Some databases contain sequences from a single organism. Others contain entries from multiple organisms, but often include the taxonomy for each entry, so that entries for a specific organism can be selected during a search using a taxonomy filter.

If you're not sure what is in the sample, Swiss-Prot is a good starting point. The entries are all high quality and well annotated. Because Swiss-Prot is non-redundant, it is relatively small. The size of the database is one factor in the size of the search space - the number of peptide sequences that are compared with a spectrum to see which gives the best match. The smaller the search space, the easier it is to get a statistically significant match. This is a very important concept and other factors that affect the size of the search space will be highlighted as we come to them.

If you think you know what is in the sample, you may want to search an organism specific database. But, you can never rule out contaminants. This can be a severe problem if you only have a handful spectra. You are interested in a human protein, so you search a human database, but your spectrum is for a peptide from a contaminant, so you get no match or a misleading match.

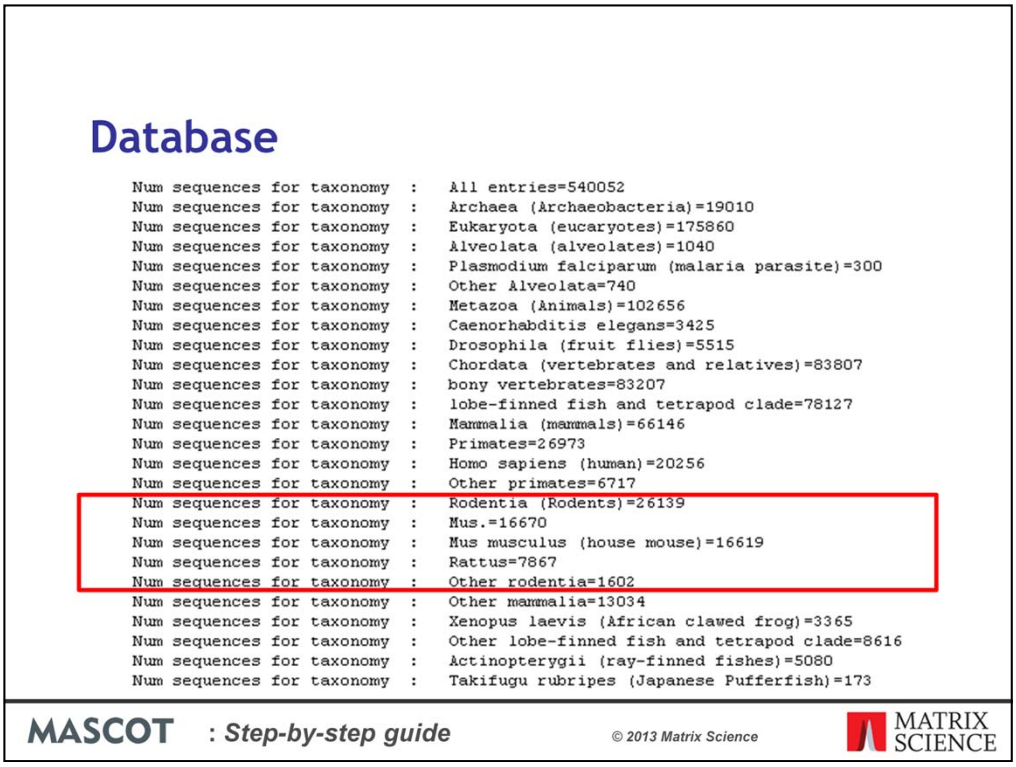
So, our second 'golden rule' is, when searching entries for a single organism, always include a database of common contaminants. This is important, even if you have a large dataset and no interest in proteins from anything other than your target organism. You may end up reporting your sample is full of human serum albumin when its really BSA or mouse keratin when its really sheep keratin from a sweater.

In the web browser search form, select your target database then hold down the control key to select an additional database of contaminants. If your search uses a taxonomy filter, that's not a problem because taxonomy is not configured for the contaminants database. All the entries will be searched, whatever the taxonomy setting.



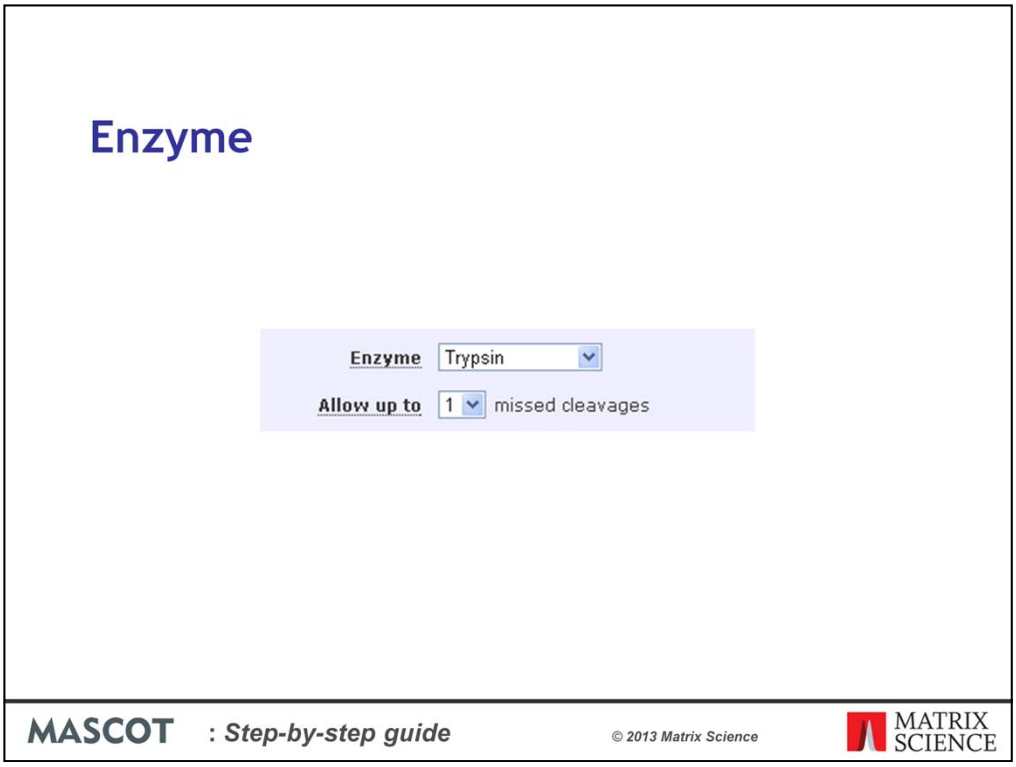
If your target organism is well characterised, such as human or mouse or yeast or arabidopsis, there may be no need to look beyond Swiss-Prot. You can get a sense of how well your organism is represented by going to http://www.uniprot.org/ and looking at the Swiss-Prot release notes, which list the 250 best represented species. On the other hand, if you are interested in a bacterium or a plant, you may find that it is poorly represented. The next place to look is one of the comprehensive protein databases, which aim to include all known protein sequences. The two best known are NCBInr and UniRef100. If the genome of your organism hasn't been sequenced, you may still be out of luck, and your best hope may be to search an EST database (Expressed Sequence Tags are relatively short nucleic acid sequences).

Here, we can see the entry in the NCBI taxonomy browser for orange, the citrus fruit. Just 94 entries for orange in Swiss_prot and only 758 in the whole of NCBInr. If this is your organism of interest, you'll probably want to search the ESTs



Don't choose a narrow taxonomy without looking at the counts of entries and understanding the classification. In the current SwissProt, for example, there are 26,139 entries for rodentia, of which all but 1,602 are for mouse and rat. So, even if your target organism is hamster, it isn't a good idea to choose 'other rodentia'. Better to search rodentia and hope to get matches to homologous proteins from mouse and rat.

Swiss-Prot is a non-redundant database, where sequences that are very similar have been collapsed into a single entry. This means that the database entry will often differ slightly from the protein you analysed. Standard database searching requires the exact peptide sequence, so you may miss some matches due to SNPs and other variants. This would be another reason to search a large, comprehensive database. However, NCBInr is 50 times the size of Swiss-Prot, so searches take proportionally longer and the search space is proportionally larger, meaning that you need higher quality data to get a significant match.



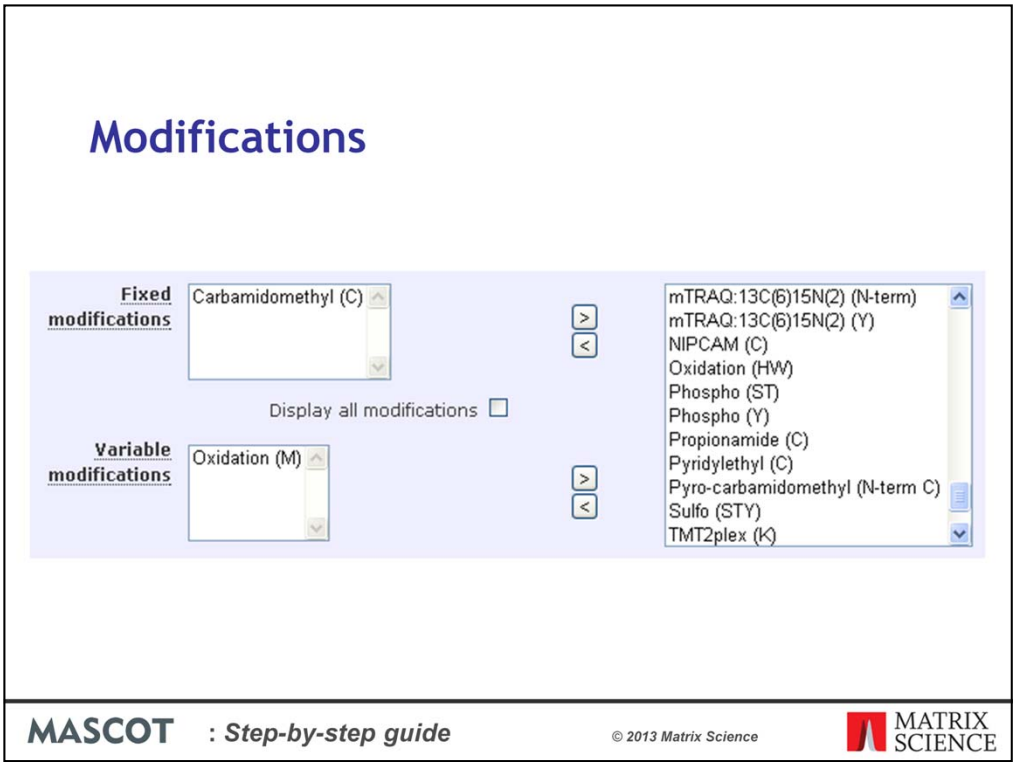
If your protein was digested using an enzyme, always choose this enzyme. Choosing a semi-specific enzyme or non-specific cleavage greatly increases the search time and the search space, which will almost certainly cause a net reduction in the number of matches. The error tolerant search, to be discussed shortly, is a much better way of finding non-specific peptides. If you are studying endogenous peptides, such as MHC peptides, you have no choice, and enzyme 'None' will look for matches in all sub-sequences of all proteins. If you are doing top-down, and analysing the intact protein, choose NoCleave. Note that NoCleave is not the same as None; in some ways, it is the exact opposite.

The number of missed cleavages should be set empirically, by running a standard and looking at the proprotions of matches with missed cleavages. Setting this value higher than necessary just increases the size of the search space, which I hope we are now coming to recognise is a 'bad thing'.

Golden Rule #3

- If your protein was digested using an enzyme, always choose this enzyme.

We'll be flagging this up as another golden rule in the summary at the end



Modifications in database searching are handled in two ways. First, there are the fixed modifications. An example would be a the efficient alkylation of cysteine. Since all cysteines are modified, this is effectively just a change in the mass of cysteine. It carries no penalty in terms of search speed or specificity.

In contrast, most post-translational modifications do not apply to all instances of a residue. For example, phosphorylation might affect just one serine in a peptide containing many serines. These variable modifications are expensive, in the sense that they increase the search space 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. We get a so-called combinatorial explosion.

Golden Rule #4

- Only specify very abundant modifications as variable.

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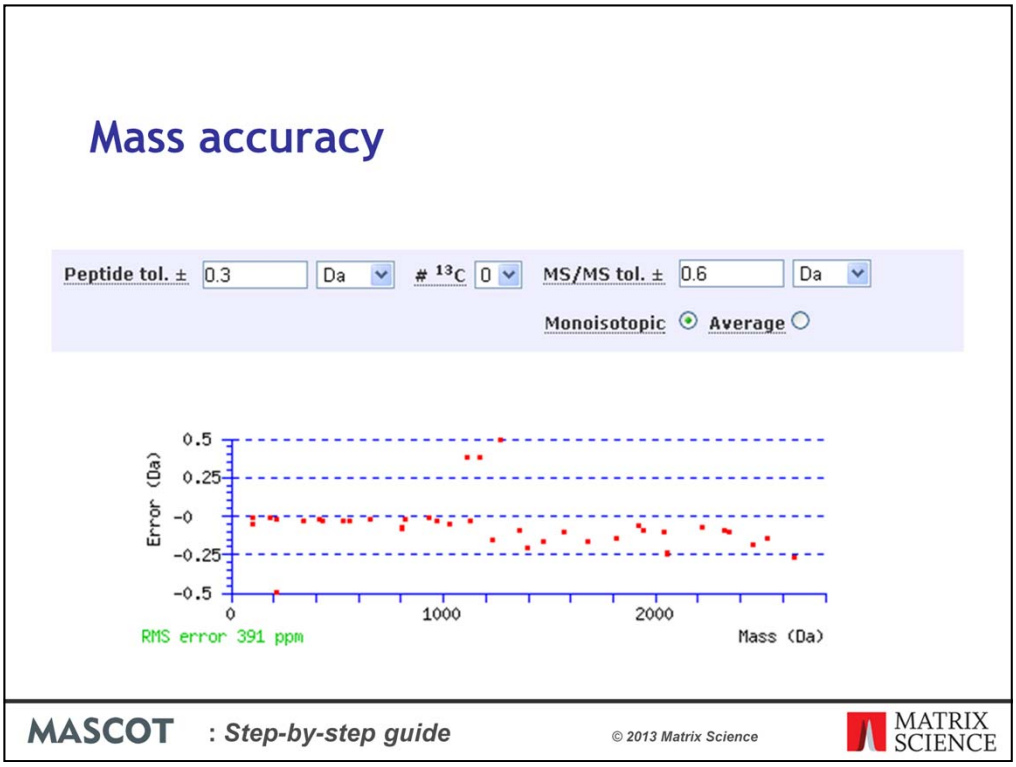
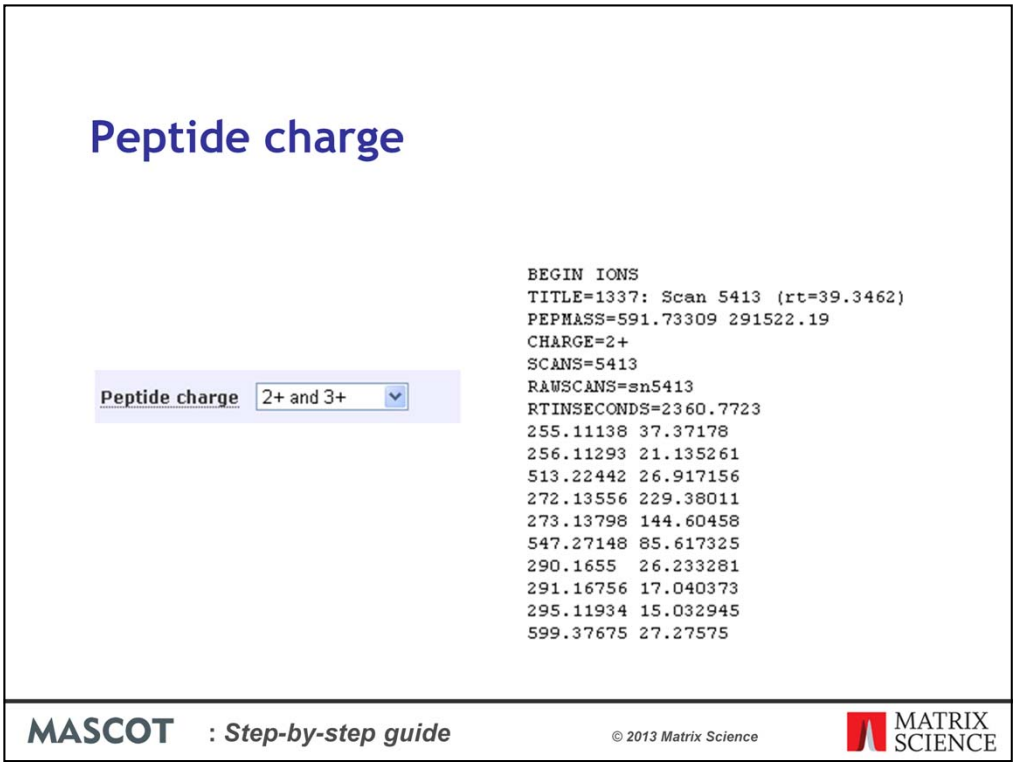
Hence, it is very important to be as sparing as possible with variable modifications. If the aim of the search is to identify as many proteins as possible, the best advice is to use a minimum of variable modifications. Most post-translational modifications, such as phosphorylation, are rare and it is much more efficient to use an error tolerant search to find them.



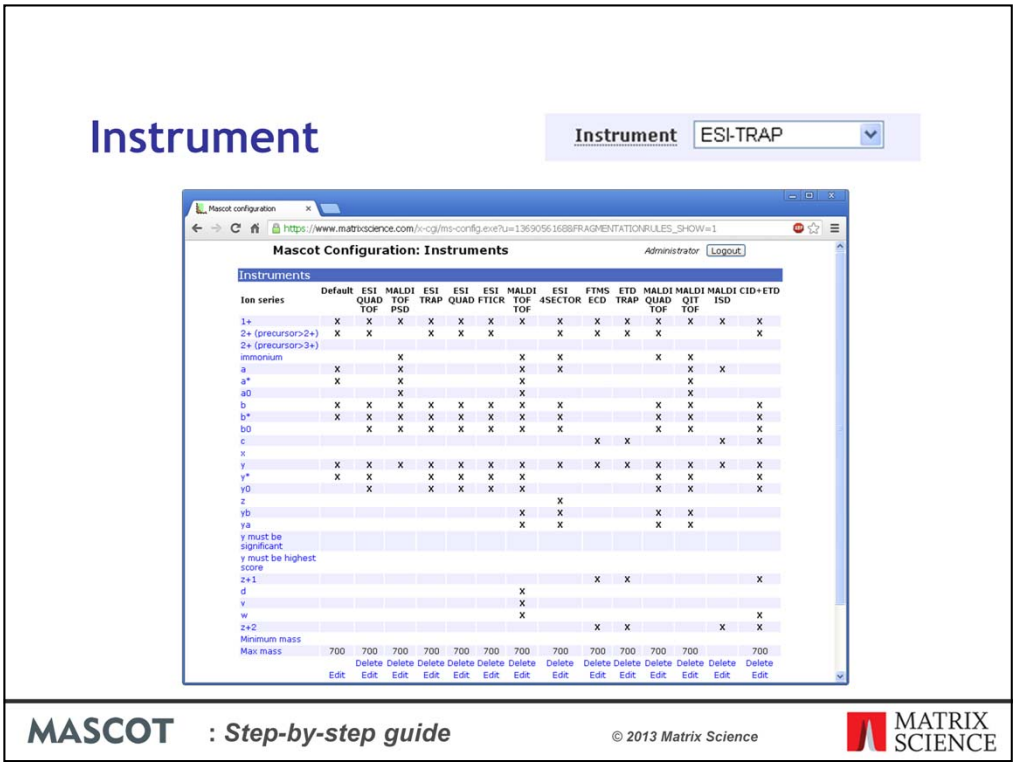
Making an estimate of the mass accuracy doesn't have to be a guessing game. The Mascot result reports include graphs of mass errors, like the one shown here. Just run a standard and look at the error graphs for the strong matches. You'll normally see some kind of trend. Add on a safety margin and this is your error estimate. The graph for precursor mass error is in the Protein View report and the graph for MS/MS fragment mass error is in the Peptide View report

Sometimes, peak picking chooses the 13C peak rather than the 12C, so the mass is out by 1 Da. In extreme cases, it may pick the 13C2 peak. The #13C control allows for this, allowing you to use a tight mass tolerance and still get a match.

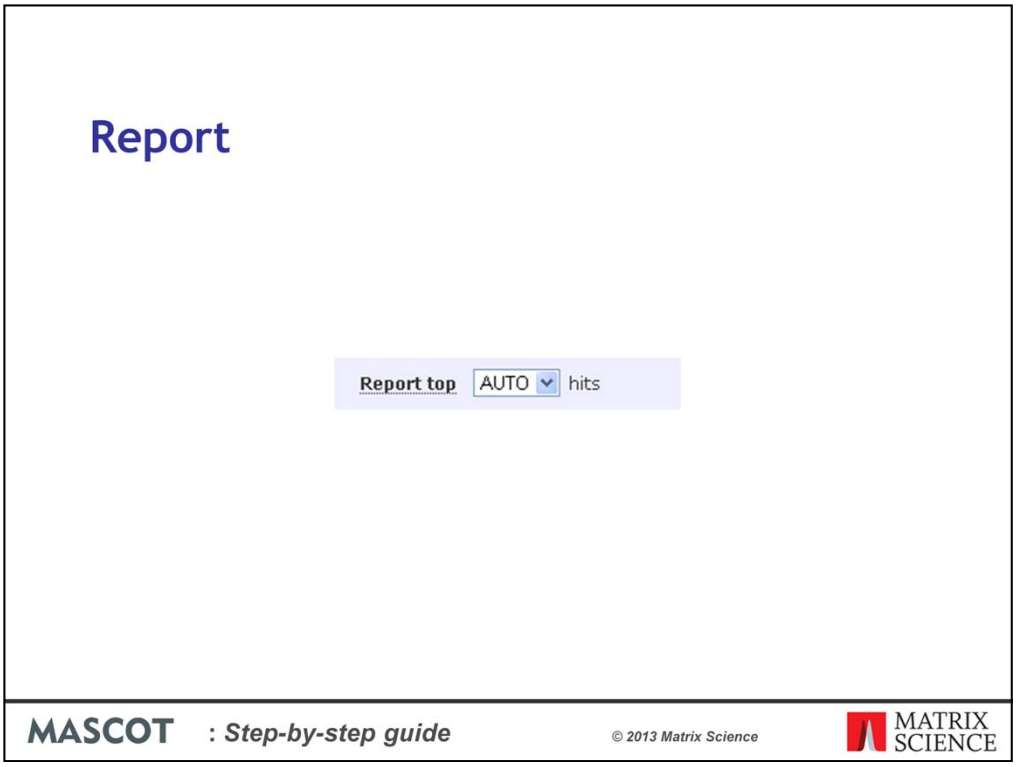
Most modern instruments produce monoisotopic mass values. You will only have average masses if the entire isotope distribution has been centroided into a single peak, which usually implies very low resolution. (If you get this setting wrong, the mass errors will be very large and show a strong trend, because the difference between an average and a monoisotopic mass for peptides and proteins is approximately 0.06%.)



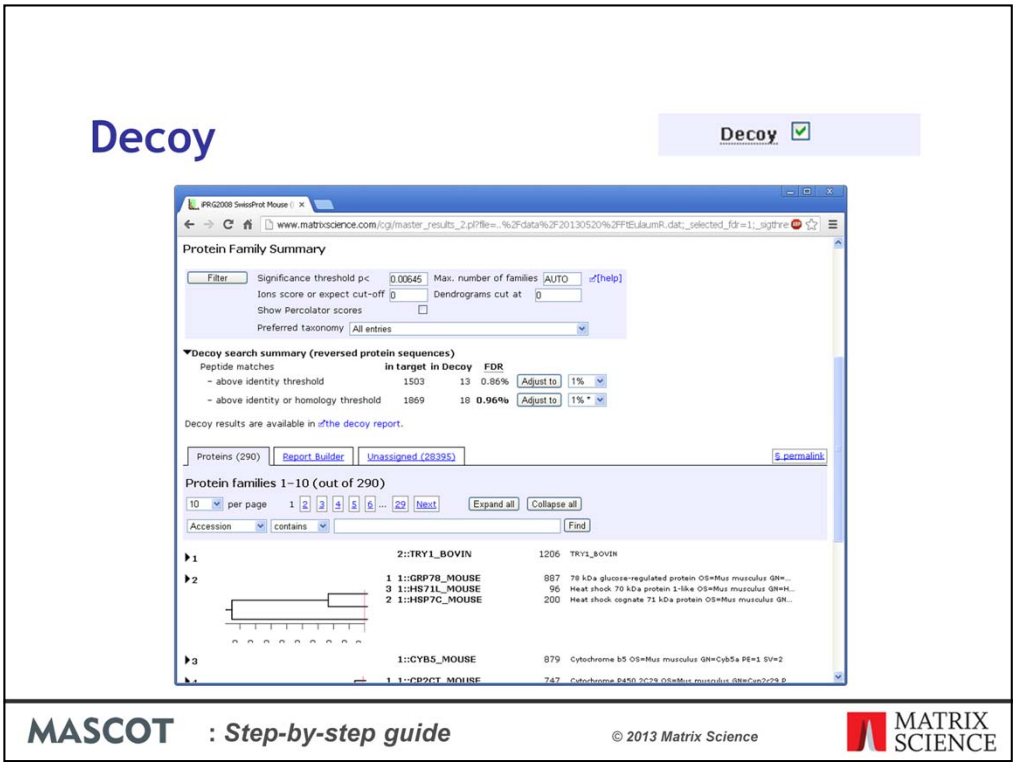
Peptide charge is a default, only used if no charge is specified in the peak list. Most peak lists always specify a charge state, so this default is never used.



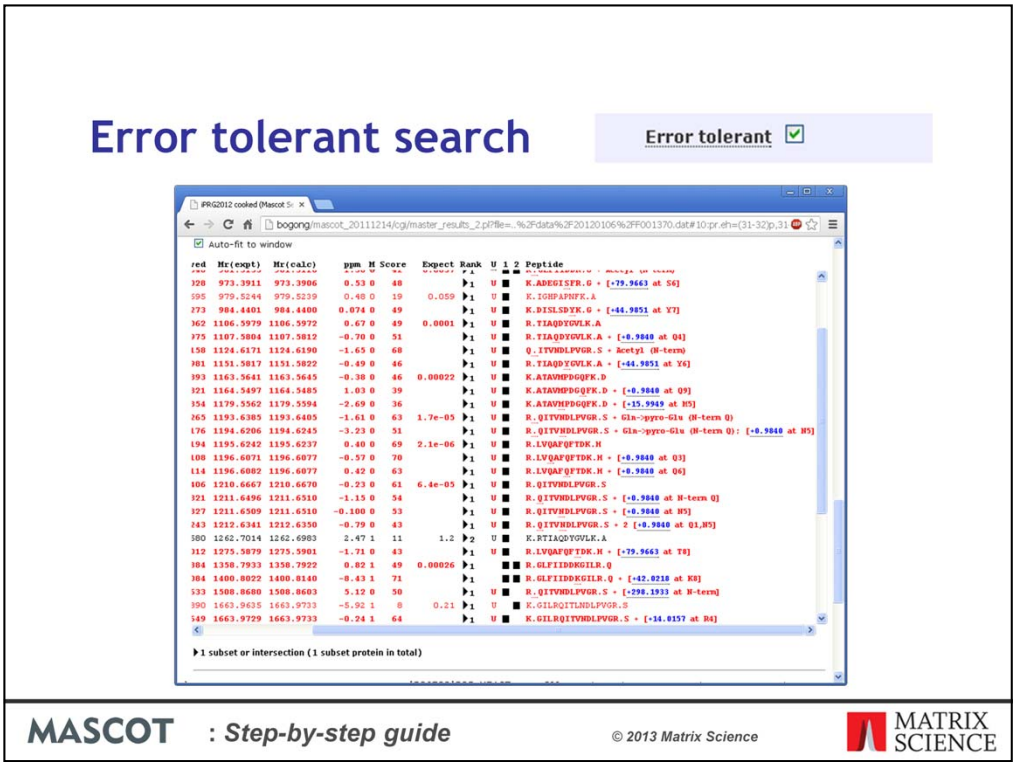
The Instrument setting determines which fragment ion series will be considered in the search. Choose the description that best matches your instrument. If you follow the label link to the help, you'll see that many of the instruments are very similar. The only serious problem is if you choose CID for ETD data or vice versa.



Report determines the maximum number of hits displayed in a search results report. Always choose AUTO to display all the protein hits containing one or more significant peptide matches. There is absolutely no point setting this to a very high value. You are just listing junk proteins, for which there is no evidence.



The decoy checkbox enables you to estimate the peptide false discovery rate as recommended by most journals. Mascot repeats the search, using identical search parameters, against a database in which the sequences have been reversed. You do not expect to get any real matches from the decoy database. So, the number of matches that are obtained in the decoy database is an excellent estimate of the number of false positives in the results from the target database. The result report gains a control that adjusts the significance threshold to achieve a peptide FDR of 5% or 1% or whatever you believe is appropriate for your work.

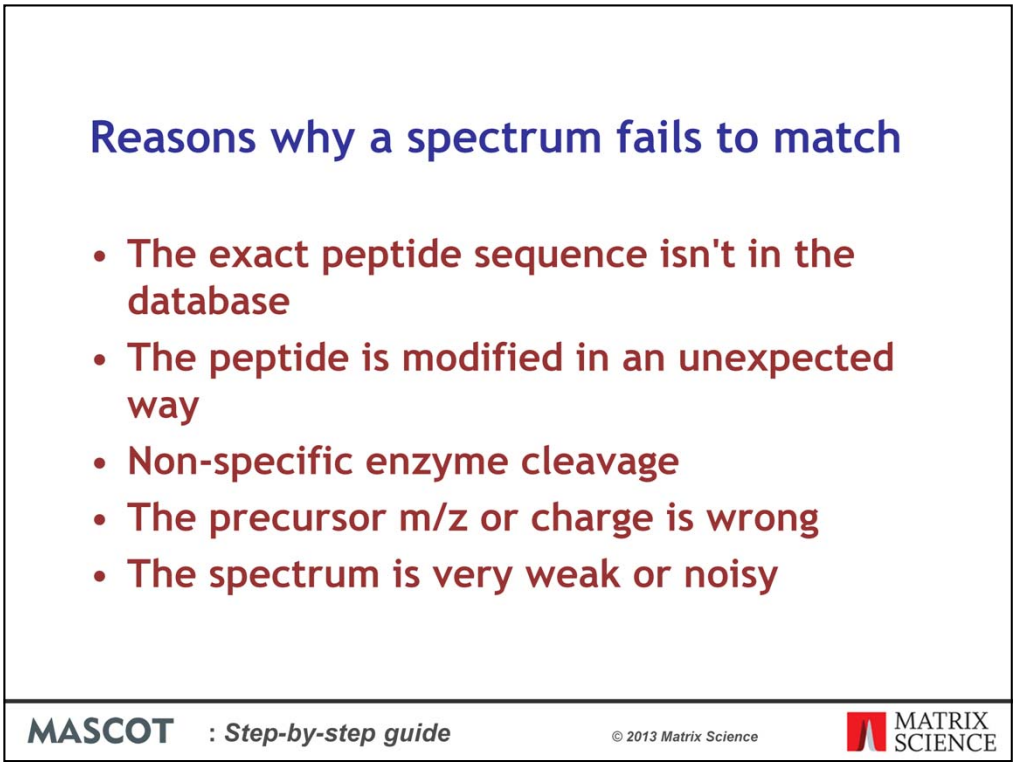


As mentioned several times already, an error tolerant search is the most efficient way to discover most post-translational modifications, as well as non-specific peptides and sequence variants. This is a two pass search, the first pass being a simple search of the entire database with minimal modifications. The protein hits found in the first pass search are then selected for an exhaustive second pass search, during which we look for all possible modifications, sequence variants, and non-specific cleavage products. Because only a small number of entries are being searched, search time is not an issue. The matches from the first pass search, in a limited search space, are the evidence for the presence of the proteins, while the matches from the second pass search give increased coverage.

Running an error tolerant search couldn't be easier; just check the box. The hard work is in studying the report, and deciding which of the modifications you believe, because there will often be a choice.

If you see a very abundant modification, best to add this as a variable modification and then search again, because the error tolerant search only catches peptides with a single unsuspected modification.

Error tolerant searching is not so useful for very heavily modified proteins, such as histones, or where there is only one peptide per protein, such as endogenous peptides



If possible, you should search a peak list containing data for as many peptides as possible. This slide lists some of the many reasons why any one spectrum may fail to give a match.

If you search one spectrum and don't get a matches, you can only resort to changing the search parameters by trial and error, which is time consuming and carries the risk of ending up with a false positive. If you search many spectra, you have a much better chance that some of them match, and the search parameters can be modified systematically, or even automatically, in an error tolerant search.

Golden rules

1. Mascot requires peak lists; you cannot upload raw data
2. Search parameters are critical and should be set on a well characterised standard
3. When searching entries for a single organism, always include a database of common contaminants
4. Only specify very abundant modifications as variable.
5. If the protein was digested with an enzyme, choose this enzyme
6. Use an error tolerant search to find post-translational modifications, SNPs, and non-specific cleavage products
7. For important work, run a target-decoy search, set the peptide FDR to 1%, and filter the proteins by requiring significant matches to 2 distinct sequences

This slide summarises the key points. If you follow these guidelines, you shouldn't go far wrong.