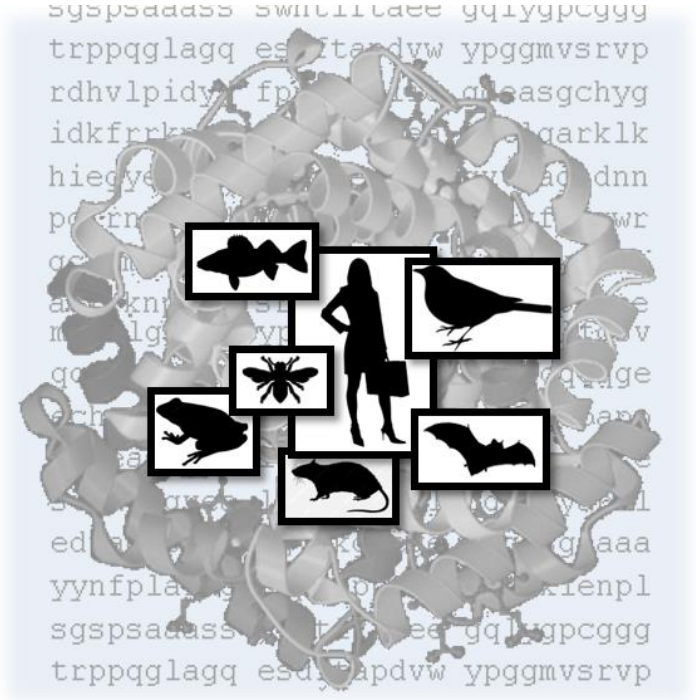
EPA/600/R-24/255

Sequence Alignment to Predict Across Species Susceptibility

(SeqAPASS)

VERSION 8.0



User Guide

Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS): User Guide
Updated 09/10/2024; Contact Carlie LaLone with Questions: LaLone.Carlie@epa.gov

Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) User Guide

Quick Notes: Use Chrome for optimal performance and PLEASE DO NOT submit more than 10 Level 1 or Level 4 queries at a time. Wait until jobs run to completion prior to submitting more.

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Background

The SeqAPASS tool has been developed to predict across species relative intrinsic susceptibility to chemicals with known molecular targets (e.g., pharmaceuticals, pesticides) as well as evaluate conservation of molecular targets from high-throughput screening assays (i.e., U.S. Environmental Protection Agency ToxCast Program) and molecular initiating events (MIEs) and early key events in the adverse outcome pathway (AOP) framework, as a means to extrapolate such knowledge across species. The term "relative" is used because it is recognized that molecular target similarity is one consideration, though an important one, for making predictions of susceptibility to a chemical. Other important considerations for susceptibility that are not evaluated using the SeqAPASS methodology include how well a chemical is absorbed, distributed, metabolized, and eliminated, life stage, and other life history traits. Also, "relative" indicates that the determination of sequence similarity between proteins is based on comparison to a single protein sequence for a specific species. Additionally, we describe "intrinsic susceptibility" as the vulnerability (or lack thereof) of an organism to chemical perturbation due to its inherent biological composition.

Cross-species comparisons of proteins can be conducted through examination of sequence and structural information, depending on how well the protein has been characterized and what is known about a chemical-protein interaction. SeqAPASS allows the user to assess various levels of protein sequence detail across species including comparisons of primary amino acid sequence (including ortholog detection), functional domain(s), individual amino acid residue positions, and structural similarity. Each level requires a greater understanding of the protein and its interaction with a chemical of interest (or similar ligand).

Because human and veterinary drugs, as well as pesticides, are designed to act specifically on well characterized molecular targets, these chemical classes have proven useful for demonstrating the utility of the SeqAPASS tool and its application to various hazard assessment/research scenarios.

The pertinent information necessary to begin a SeqAPASS query includes: the identification of a single (or multiple) query species and a query protein, which would be the molecular target(s) of interest (e.g., receptor or enzyme).

The SeqAPASS algorithms mine, collect, and collate information from the National Center for Biotechnology Information (NCBI) protein database (http://www.ncbi.nlm.nih.gov/protein/), conserved domains database (http://www.ncbi.nlm.nih.gov/cdd/), taxonomy database (http://www.ncbi.nlm.nih.gov/taxonomy/), strategically utilizes the Stand-Alone Basic Local Alignment Search Tool for proteins (BLASTp) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=Download and the Constraint-based Multiple Alignment Tool (COBALT)

(http://www.st-va.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi).

Advanced users can request Level 4 access for protein structure generation and alignment to gather additional lines of evidence toward conservation. Tools used for structural evaluation include: Iterative Threading ASSEmbly Refinement (I-TASSER, https://zhanggroup.org/I-TASSER/) for creation of protein structural models, AlphaFold (https://AlphaFold.ebi.ac.uk/), iCn3D for viewing and manipulating protein models, and other sources using PDB formated protein structures for structural alignment using TM-align (https://zhanggroup.org/TM-align/).

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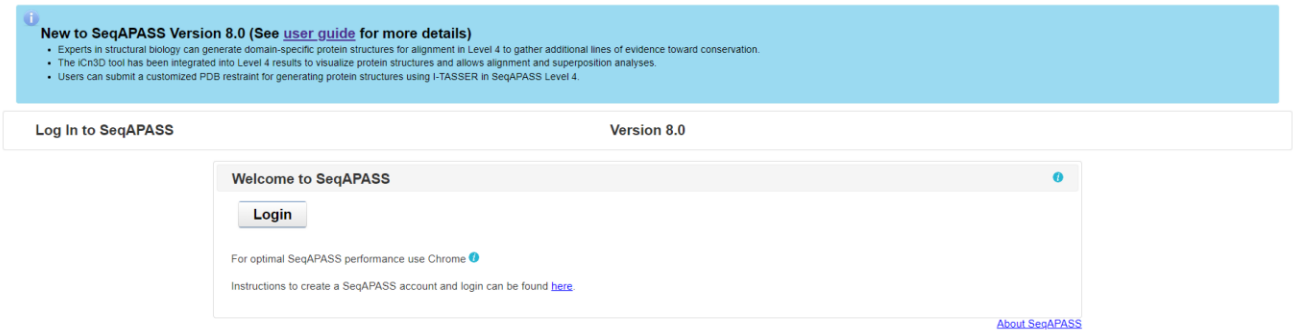
Accessing SeqAPASS

For optimal SeqAPASS performance use **Chrome**

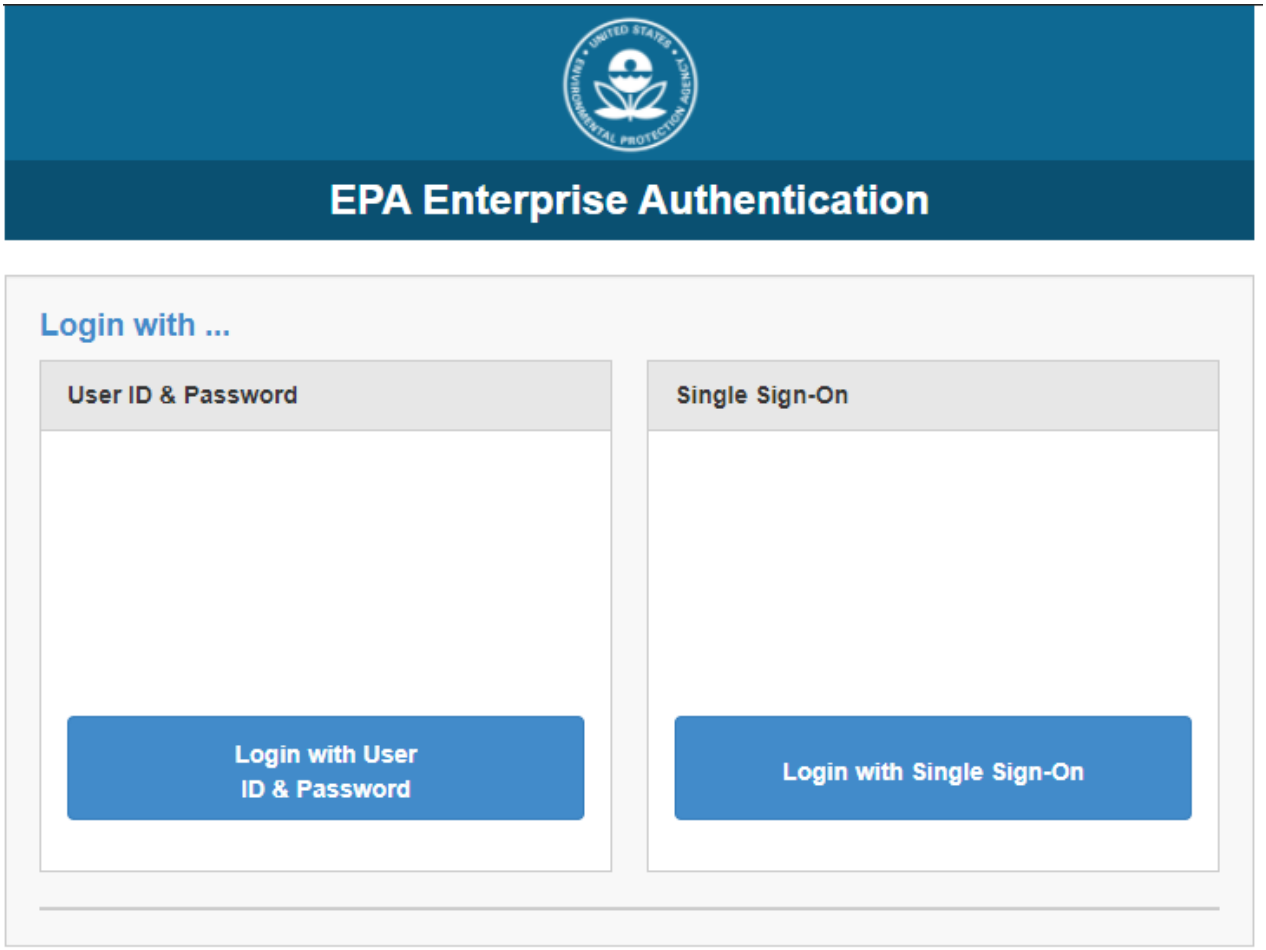
Access SeqAPASS using the following URL: **https://www.seqapass.epa.gov/seqapass/**

Returning Users

Click "Login"

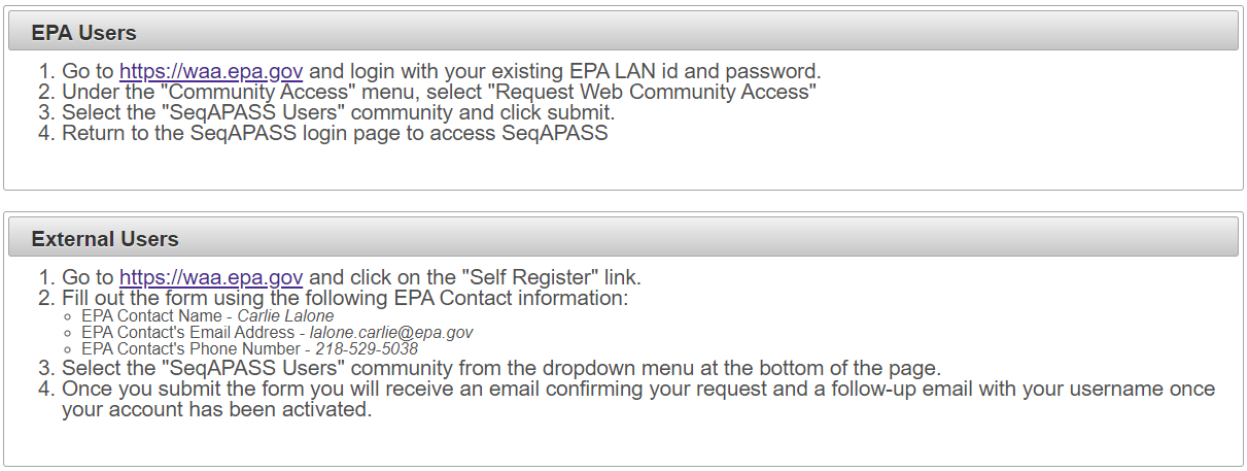


Select either "Login with EPA LAN User ID & Password" or "Login with Single Sign-On".



First time users

To request a username and password to access the SeqAPASS tool, select "here" below the login and follow the directions on the next page. The directions are different for the internal EPA user versus the external non-EPA user; however, the user type does not limit access to the tool. Everyone that requests an account will be given one in a timely manner. Individual accounts allow users to store all previous SeqAPASS runs. Once the user has obtained their username, external users will select "Login with EPA LAN User ID and Password."



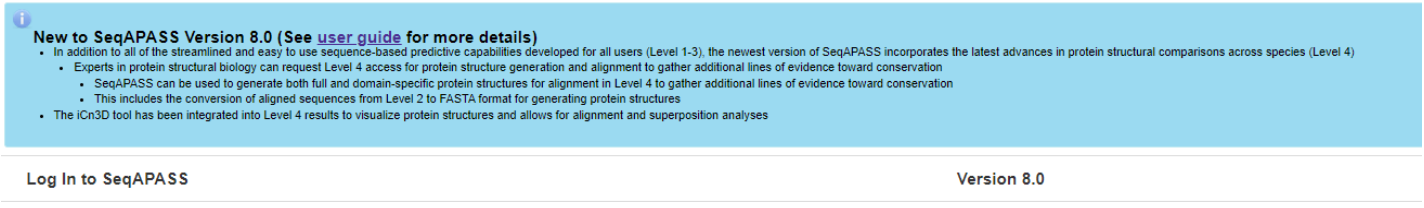
On the Log in screen the user will provide the necessary Login information:

> EPA User: EPA LAN User ID & Password ***or*** Login with Windows Single Sign-On
> External User: Username and Password

Upon creating your password, login to SeqAPASS as described above for *Returning Users*. To change a password at any time, go to waa.epa.gov and select "User Profile" to reset. The user will then use the new password to login.

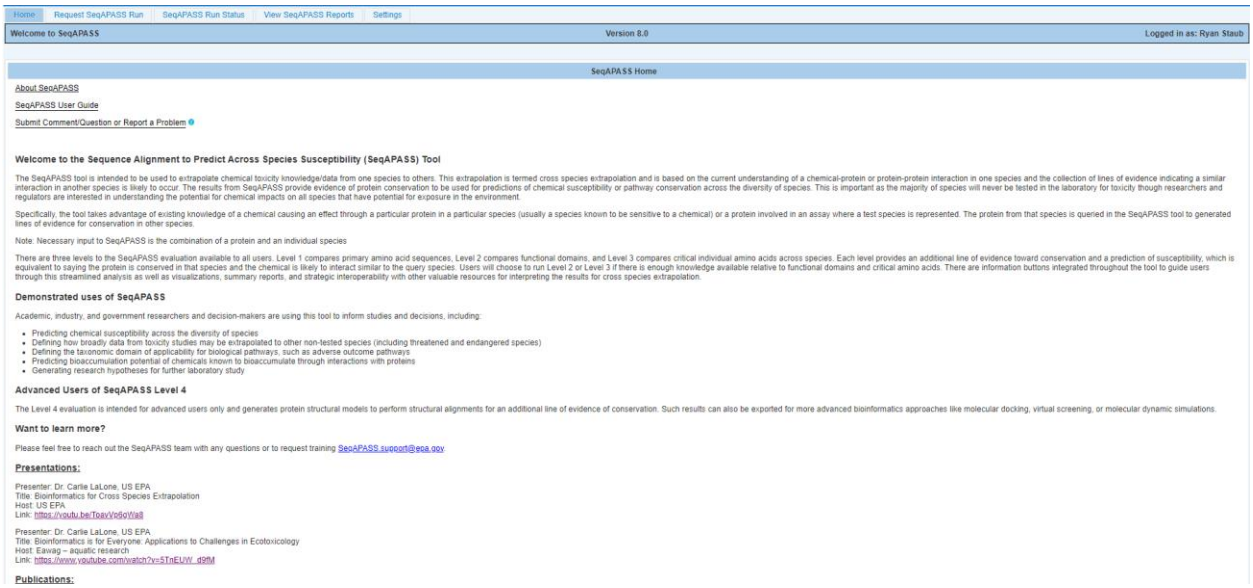
Messages from the SeqAPASS development team

Look for messages about planned version releases, data updates, and/or fixes to the SeqAPASS tool. These will occasionally be displayed below the SeqAPASS banner when the development team has information to share with SeqAPASS users.



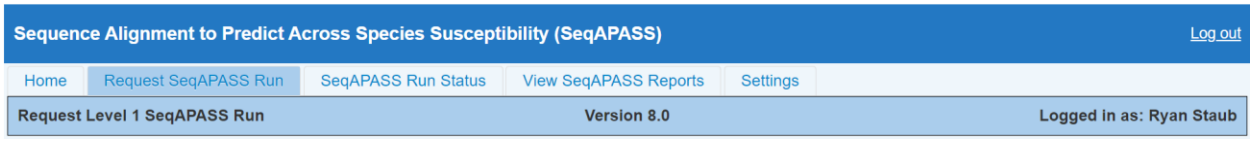
SeqAPASS Home Tab

The "Home" tab indicates who is logged in to the tool (right-hand of the screen) and contains links to obtain information about the SeqAPASS tool (About SeqAPASS), including contact information for support and references to published articles describing the SeqAPASS tool and its applications. Other relevant references to databases and tools are also referenced. A link to the SeqAPASS User Guide can also be found on this page. To Submit a Comment/Question click on the "Submit Comment/Question" link to email the developer. The "Log out" icon in upper right-hand corner of screen can be clicked at any time to log out. "Information" buttons are present throughout SeqAPASS to give the user additional information or instruction regarding features and functionality of the tool. "Exit" buttons are also present by each external (non-EPA) link that takes the user to a page ***NOT*** maintained by the EPA.



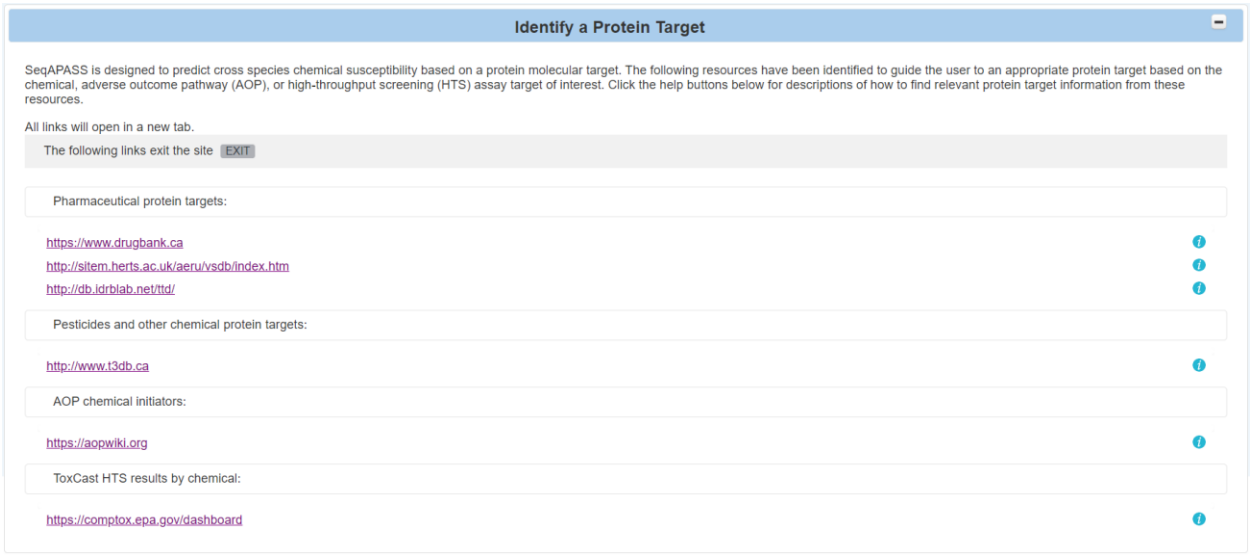
Request SeqAPASS Run Tab

Clicking the "Request SeqAPASS Run" tab opens a page to enter the query information necessary for a SeqAPASS run. Each section of the "Request SeqAPASS Run" will be described below:



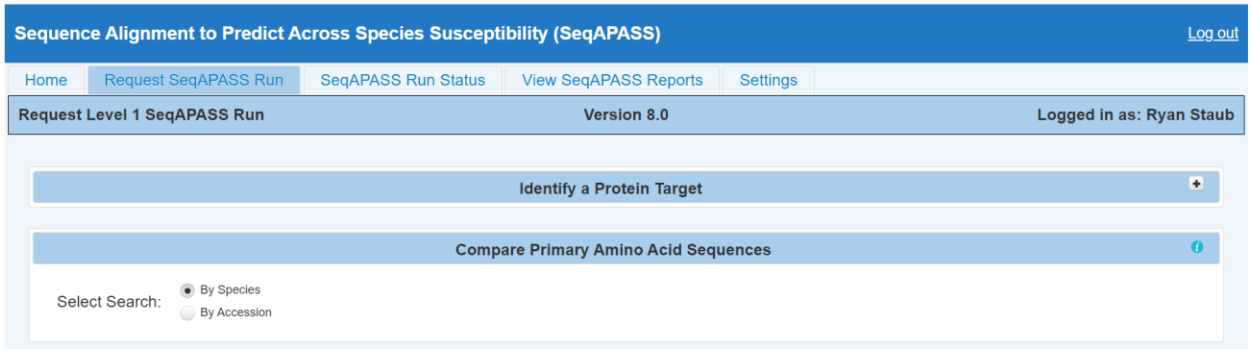
Identify a Protein Target

SeqAPASS is designed to predict cross species chemical susceptibility. Protein targets are often decided based on chemical, adverse outcome pathway (AOP), or high-throughput screening (HTS) assay target. Resources have been provided, as links, to aid the user in searching for appropriate protein targets and can be accessed by selecting the drop-downs found in the "Identify a Protein Target" box.



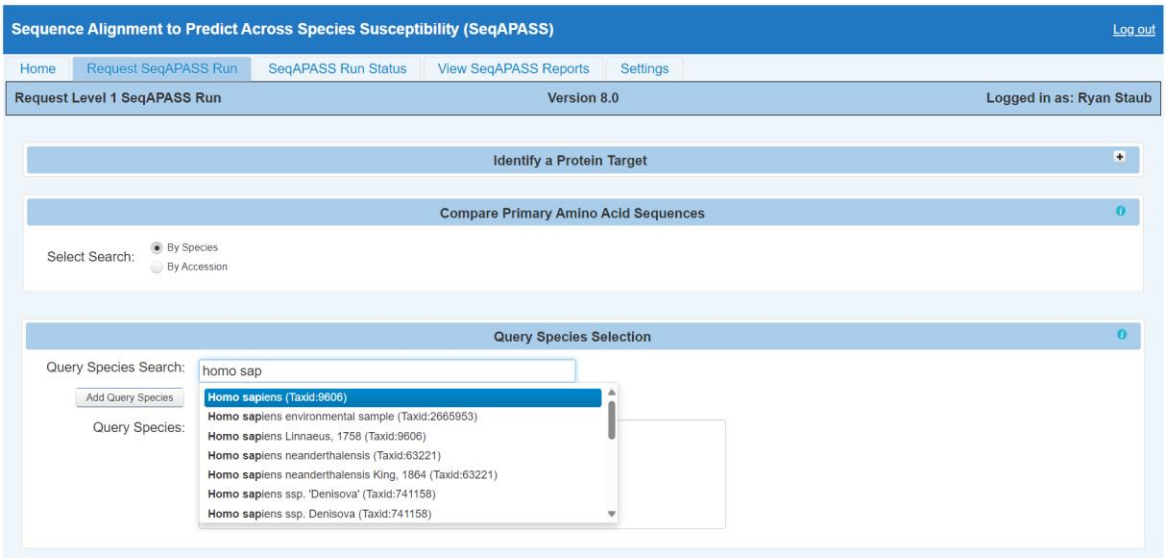
Select Search

There are two options for entering query information: "By Species" or "By Accession" (See radio buttons to the right of "Select Search"). Selecting "By Species" will allow the user to enter text and select from a dropdown list of species and then select a protein from any sequence available for that species in the NCBI protein database. Selecting "By Accession" allows the user to enter a NCBI protein accession.



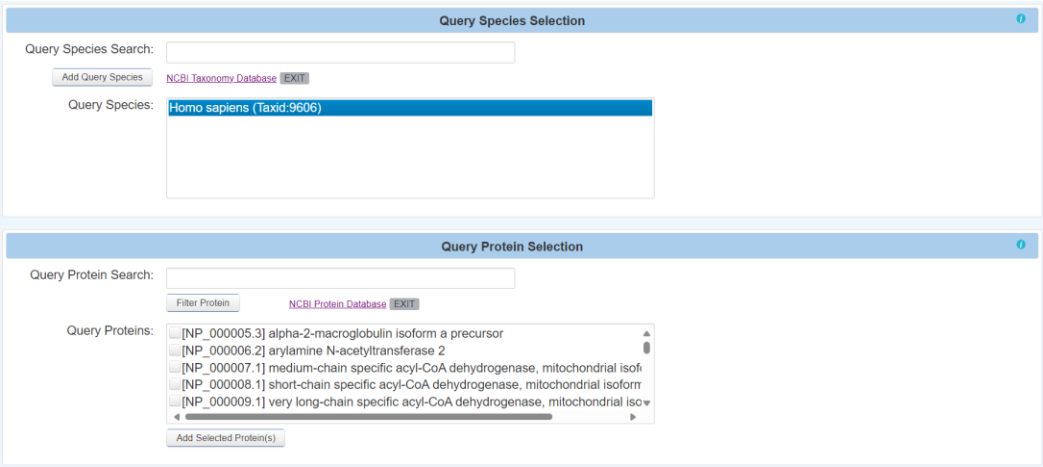
Query "By Species"

Type the name of the query species of interest in the "Query Species Search" text box. The species common name, scientific name, or Taxid (ID number derived from the NCBI taxonomy database) may be typed into the search bar. This is the species you would like to compare all other species to. The search bar has an auto-complete function and will generate a list of species with corresponding Taxid. When text is typed into the search bar, the auto-complete function queries the database in the order of "starts with" then "contains." If an integer is typed in the search bar the auto-complete function queries the database in the order of "Taxid", "starts with", then "contains."

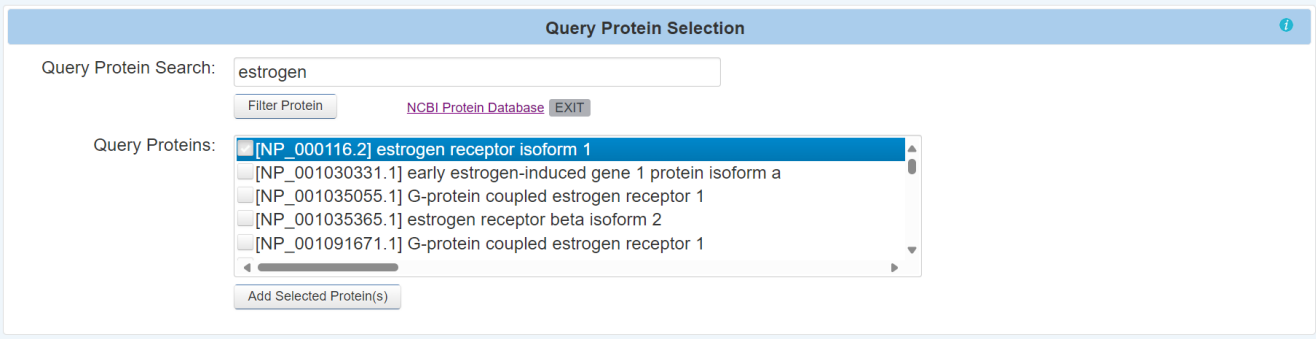


Note: The user can also use the NCBI taxonomy database to identify query species using the NCBI link on the right-hand side of the "Add Query Species" button.

Select species of interest by clicking on the name in the drop-down box. Once species is selected, click "Add Query Species" button. This advances the species of interest to the "Query Species" box and fills the "Query Proteins" box with all available protein sequences for that species from the NCBI protein database (although the box only displays the initial 200 proteins/species based on lowest numerical accession number). The protein list includes the protein NCBI accession, protein name, and species scientific name.

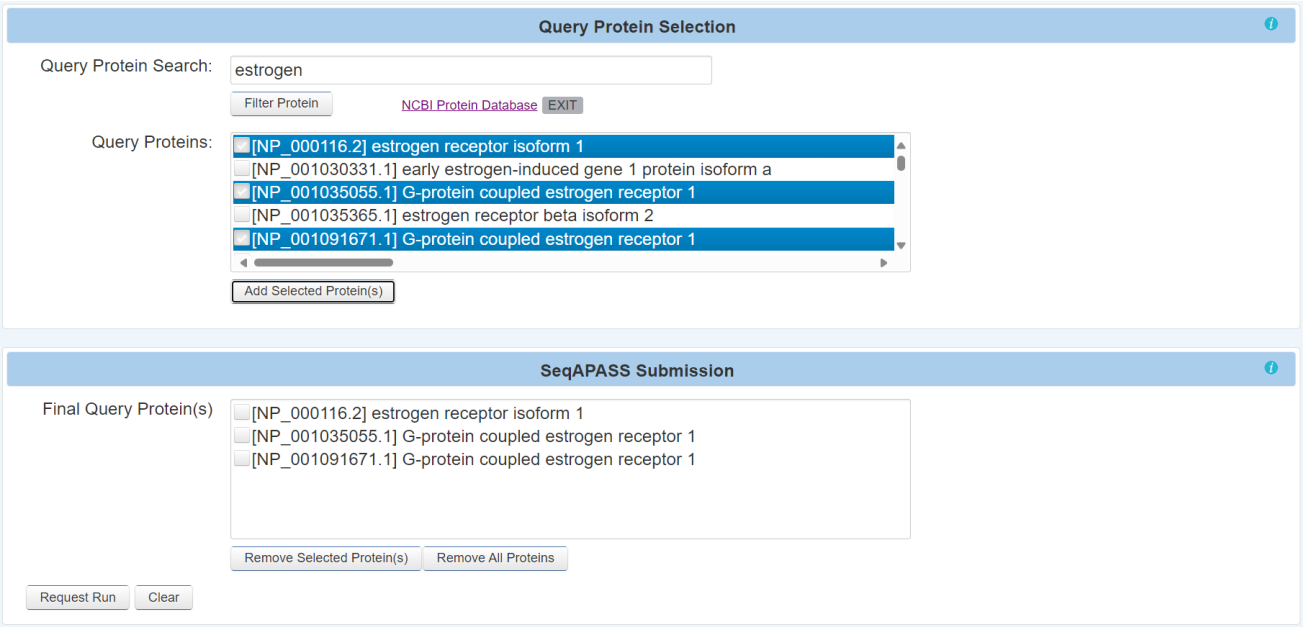


To filter the query protein list, type the query protein name or partial name in the "Query Protein Search" box and click the "Filter Protein" button. This action will filter the protein list in the "Query Proteins" box to only display proteins that contain the user defined text (this search query does not contain an autofill feature due to the filter feature). Proteins will be listed in alphabetical order based on NCBI accession Example: typing "estrogen" retrieves all proteins that contain the word "estrogen" in the protein name (the user can scroll to identify proteins of interest).

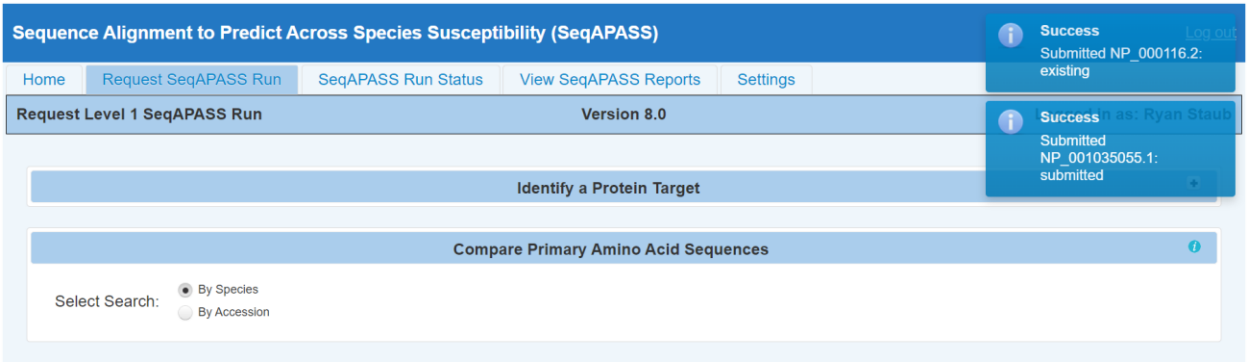


Note: To explore details associated with a protein of interest, click the "NCBI Protein Database" link to the right of the "Filter Protein" button to open NCBI proteins database (See **SeqAPASS Documentation** section of user guide for details about searching for query proteins using NCBI database).

Highlight the protein or proteins of interest in the "Query Proteins" box and click "Add Selected Protein(s)" button. This moves the protein(s) of interest to the "Final Query Protein(s)" box. To remove proteins from the "Final Query Protein(s)" box highlight those to be removed and click the "Remove Selected Protein(s)" button. Select "Remove All Proteins" to discard all proteins from "Final Query Protein(s)" box. The clear button removes all information previously entered on the "Request SeqAPASS Run" page.

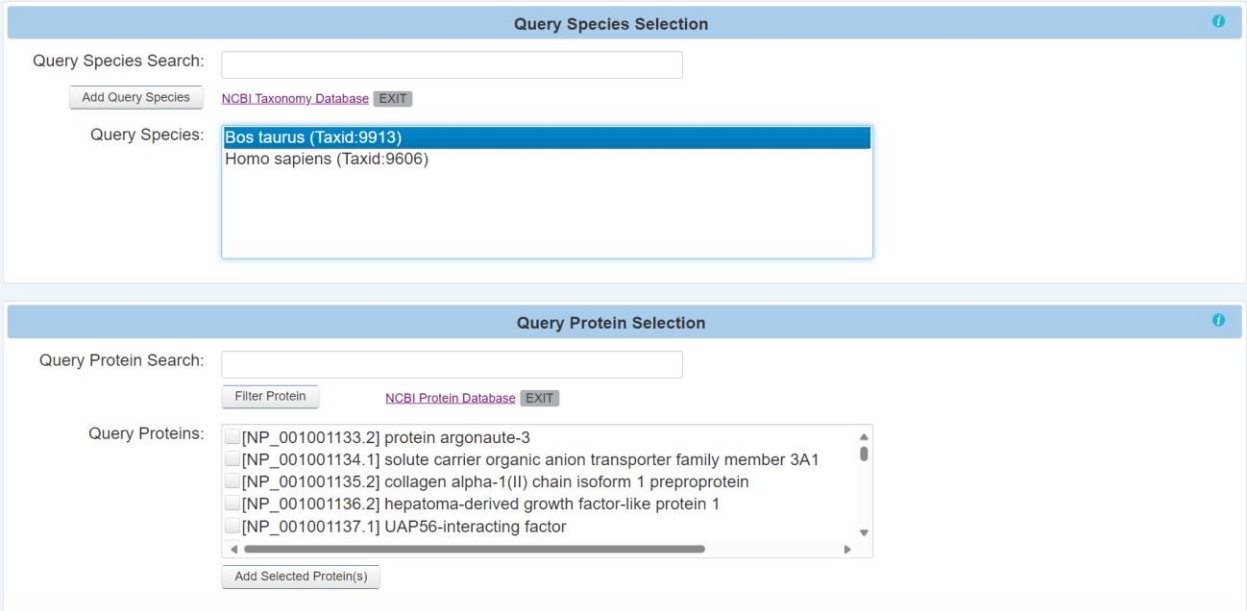


Once the user identifies the protein(s) to be queried, select "Request Run." A message will briefly appear in upper right-hand corner of the screen for 10 seconds to alert the user of the request status.



Multiple proteins can be added to the final list for multiple SeqAPASS runs. If another query species is desired, return to "Query Species Search" to select the next species. Follow the process described above for selecting the proteins associated with this species. The proteins populated in the "Query Proteins" box will always be associated with the species highlighted in the "Query Species" box.

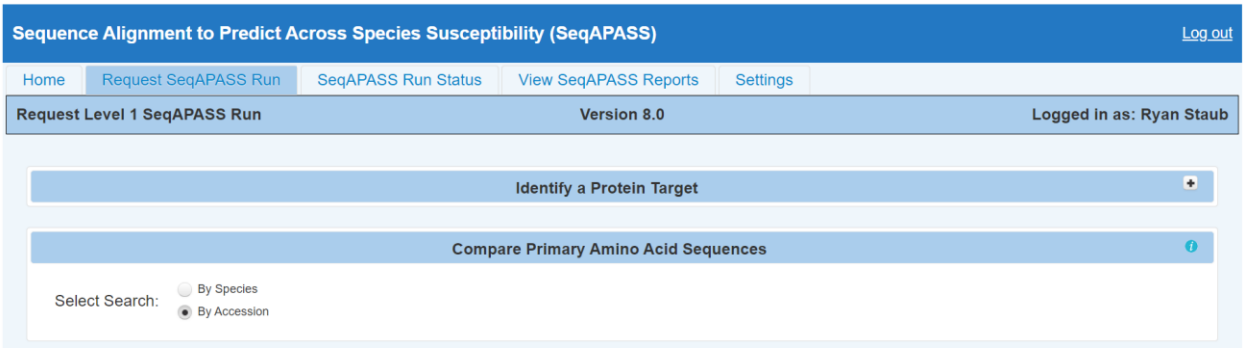
Note: In the current version of SeqAPASS, ***PLEASE do not request more than 10 query proteins at a time*** to avoid longer wait times for the completion of a run.



Note: A user may check the progress of the run by clicking on the "SeqAPASS Run Status" tab. (See **SeqAPASS Run Status** section of the user guide for more information)

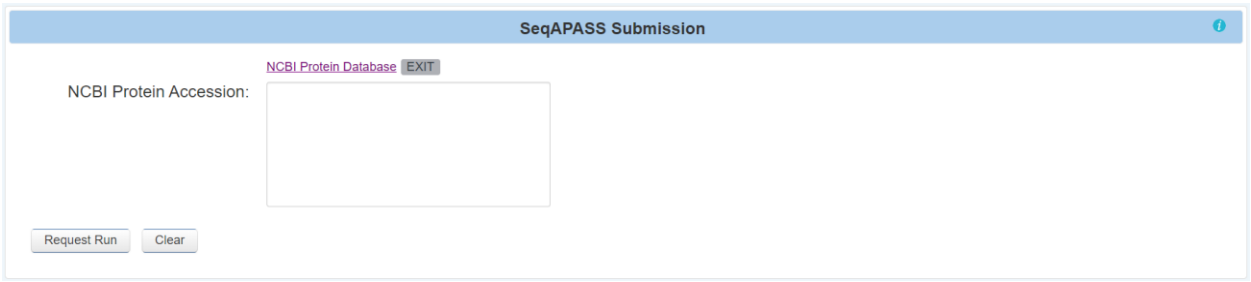
Query "By Accession"

Users familiar with the NCBI database can utilize NCBI protein accessions (e.g., NP_000116.2) to query the SeqAPASS tool. This is done by selecting the "By Accession" radio button to the right of the "Select Search" text on the "Request SeqAPASS Run" page.

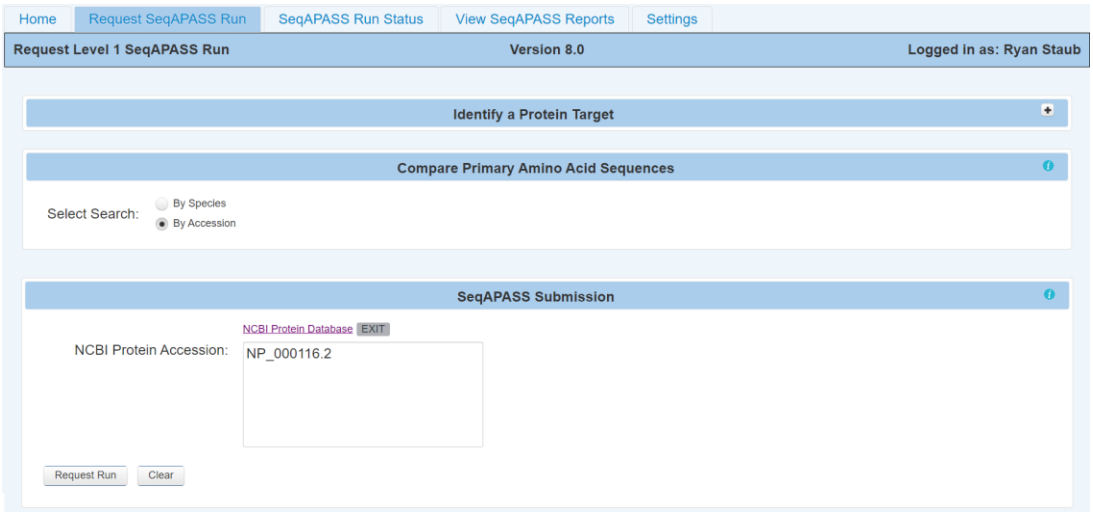


Upon selecting the "By Accession" radio button, a new query page will be displayed. Type the NCBI protein accession (e.g., NP_000116.2) for the protein of interest (this Accession comes from the NCBI protein database; See "**SeqAPASS Documentation**" for details) in the "NCBI Protein Accession" box. If desired, more than one NCBI Accession may be entered into the "NCBI Protein Accession" box by clicking the enter key after each additional NCBI Accession entry.

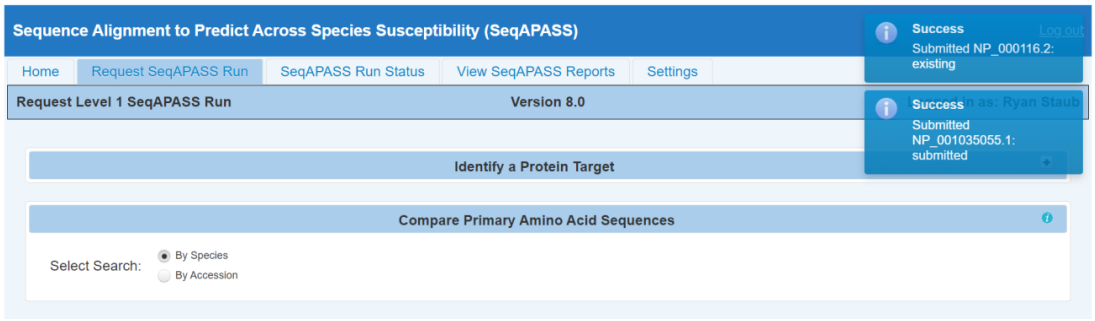
Upon clicking the "NCBI Protein Accession" text box, a pop-up message will appear in the middle of the text box, to provide an example for the proper format of Accessions to be entered.



Note: To avoid longer wait times for the completion of a run, in the current version of SeqAPASS, ***please do not request more than 10 NCBI Accessions at a time***.



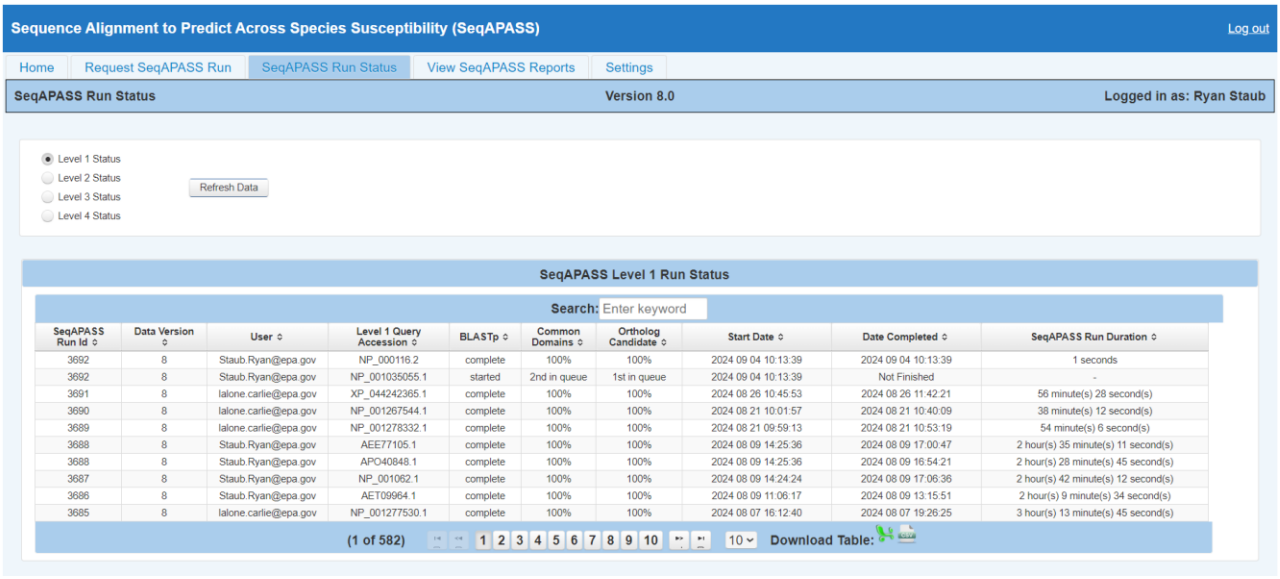
After the NCBI accession(s) of interest have been typed in the "NCBI Protein Accession" box, click the "Request Run" button. To remove proteins from the "NCBI Protein Accession" box click the "Clear" button. A message will briefly appear in the upper right-hand corner of the screen to alert the user of their run request status.



Note: All NCBI Accessions can include the version number (one digit after the decimal place, e.g., NP_000116.2). Otherwise, if the version is not included, the most recent version of the accession will be queried automatically.

SeqAPASS Run Status

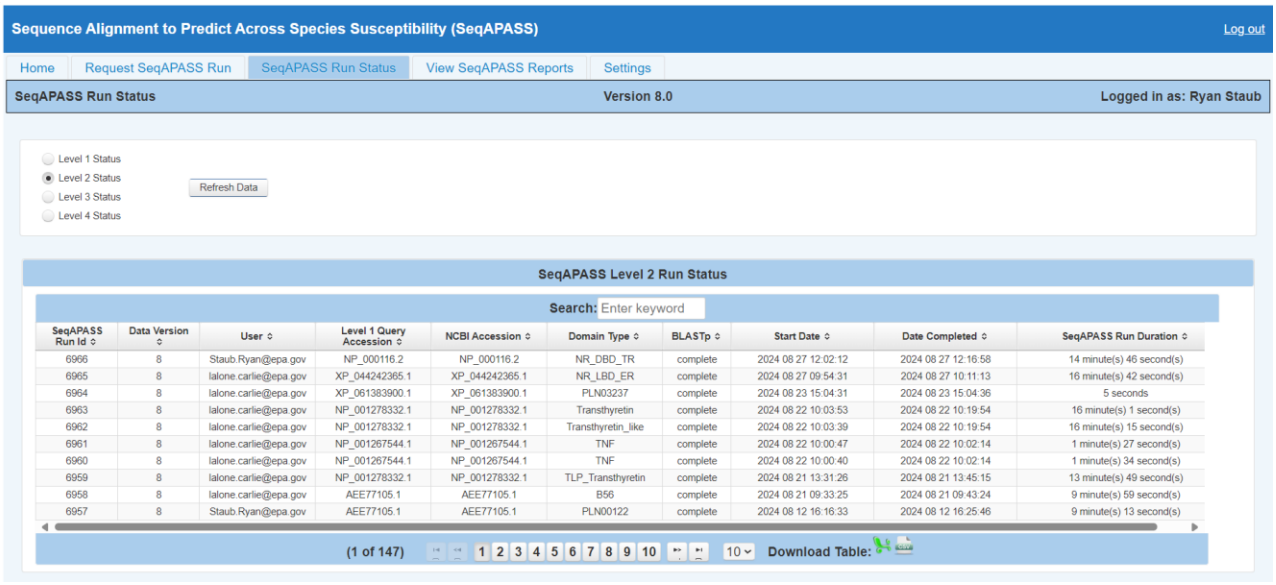
Level 1 SeqAPASS (primary amino acid sequence comparisons) status is displayed as the default. The Accession in the column "Level 1 Query Accession" is that selected and queried by the user. For a query to finish it must display "complete" in the BLASTp column, 100% in the "Common Domains" column, and 100% in the "Ortholog Candidate" column. The "Common Domains" column displays the % completion for running Reverse Position Specific (RPS)-BLAST (Default E-value of ≤0.01) on the Accessions from the Level 1 Full Report. RPS-BLAST, and therefore "Common Domains" status, will take the longest to complete. The "Ortholog Candidate" column displays the % completion for running a reciprocal best hit BLAST evaluation for each hit sequence. The status for the "BLASTp" column is described as "started," "analyzing," or "complete." If the user's successfully submitted query has entered the run queue, the position of the submitted query in the queue will be indicated in the column (e.g., 2nd in queue). The "Common Domains" and "Ortholog Candidate" columns will also describe the position of the user's submitted query in the run queue. Once the run has begun processing, the % completed for RPS-BLAST or reciprocal best hit BLAST, respectively, will be displayed. Please see example below:



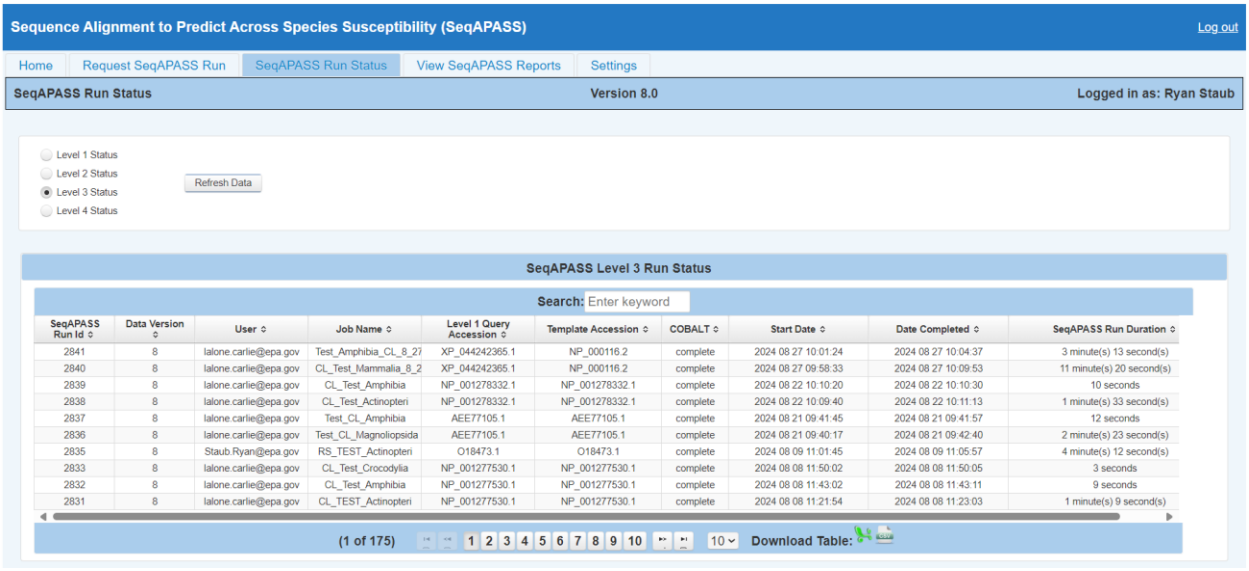
The user can view the status of requested SeqAPASS runs. Each Run is assigned a unique "SeqAPASS Run Id." A ***Run*** is considered a query that was requested either individually or as a batch in the "Request SeqAPASS Run" tab. The user can view run start and end dates/times, and the duration of the run. (See **Search, View, and Download Data Tables** section of user guide for more information). The "Data Version" column indicates which version of NCBI data is being used (See "About" page for details on Data Versions)

The user is also able to view the status of Level 2 (Functional domain(s)), Level 3 (individual amino acid residue alignments), and Level 4 (FASTA, I-TASSER, and TM-align results).

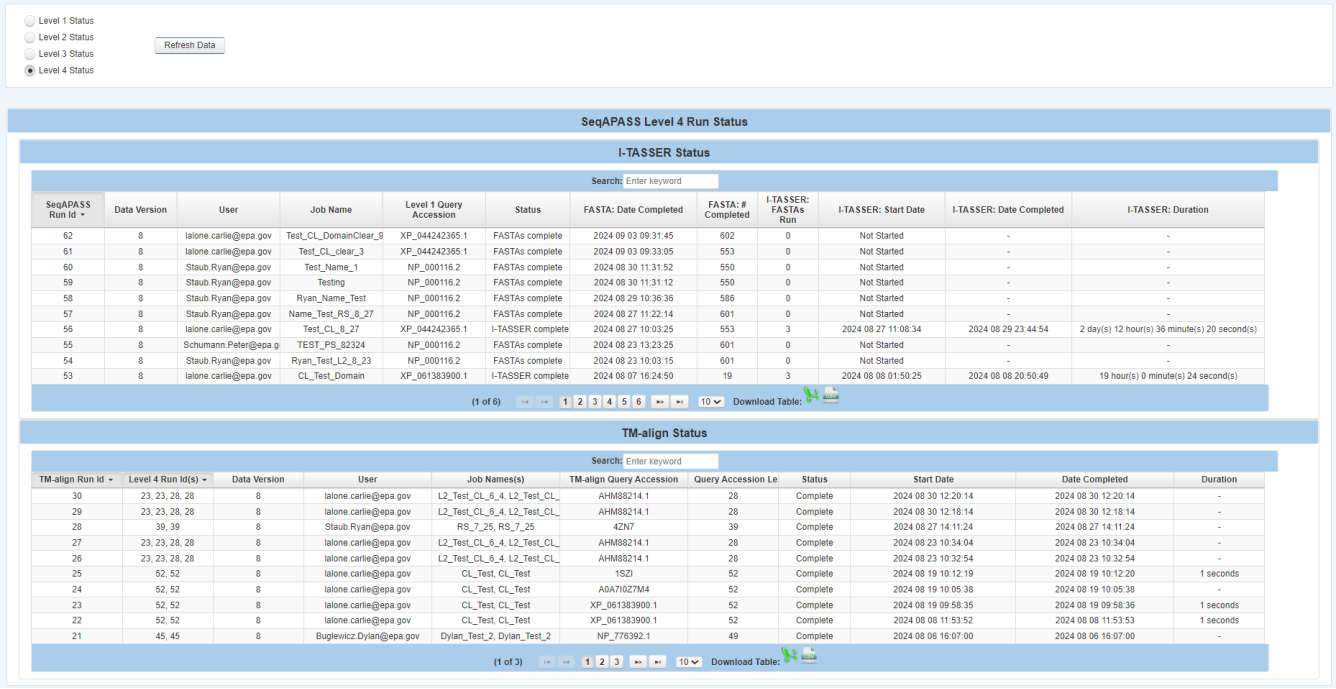
View Level 2 Status by selecting the radio button. Also, while viewing the page, the user can click the "Refresh Data" button to refresh the data. "Level 1 Query Accession" column displays the NCBI accession selected and queried by the user. Please see below:



View Level 3 Status by selecting the radio button. “Level 1 Query Accession” column displays the NCBI accession selected and queried by the user. The “Job Name” is the user defined name chosen to describe the Level 3 alignment. Also, while viewing the page, the user can click the “Refresh Data” button to refresh the data. Please see below:



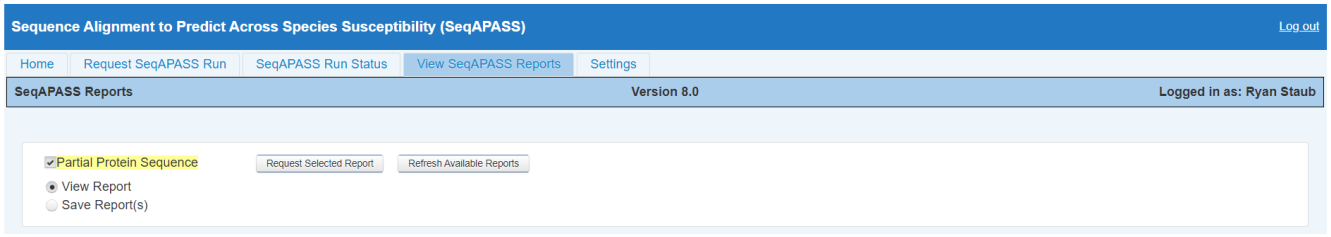
View Level 4 Status by selecting the radio button. “Level 1 Query Accession” column displays the NCBI accession selected and queried by the user. The “Job Name” is the user defined name chosen to describe the Level 4 FASTA Prioritization. While viewing this page the user may click the “Refresh Data” button to refresh the data. This page will display the status of each component of the Level 4 jobs from FASTA, I-TASSER, and TM-align.



To return to previous tabs click on “Home,” “Request SeqAPASS Run,” or “SeqAPASS Run Status” tabs.

View SeqAPASS Reports Tab

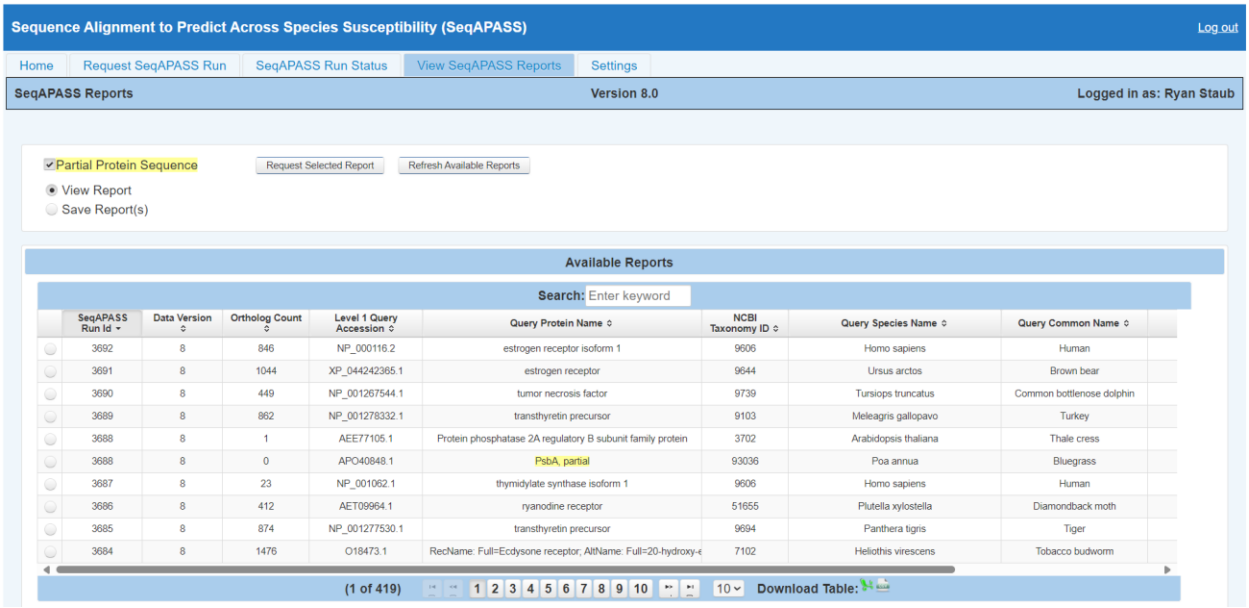
The “View SeqAPASS Reports” tab provides a table of completed SeqAPASS runs. From this page the user can choose to either “View Report” or “Save Report(s).”



The completed runs, by default, are listed in the order in which they were completed, with the most recent runs at the top. The table includes information for each run, such as SeqAPASS Run ID (unique for every run regardless of if it is the same protein/species combination ran twice), Data Version, Ortholog Count (number of orthologs detected from the aligned hit sequences in Level 1; see Detailed Documentation page 79), NCBI Accession, Query Protein Name, taxonomy information for the query species, and the date/time of run completion.

While viewing the page, the user can click the “Refresh Available Reports” button to refresh the table with additional completed runs. Partial protein sequences are highlighted in yellow as illustrated in the

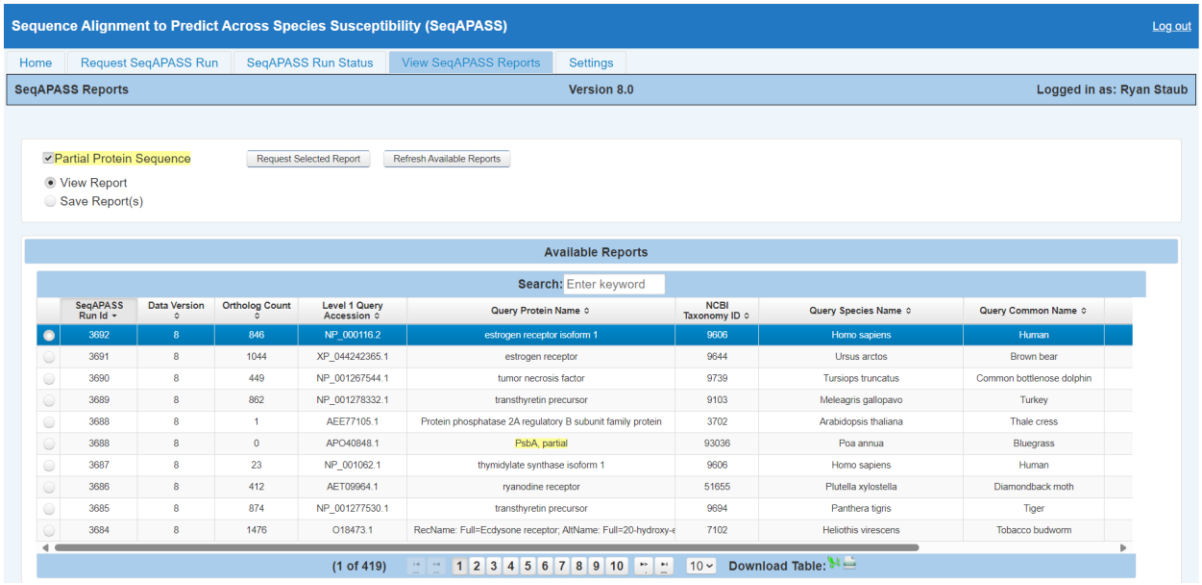
example below. (See **Search, View, and Download Data Tables** section of user guide for more information).



View Report

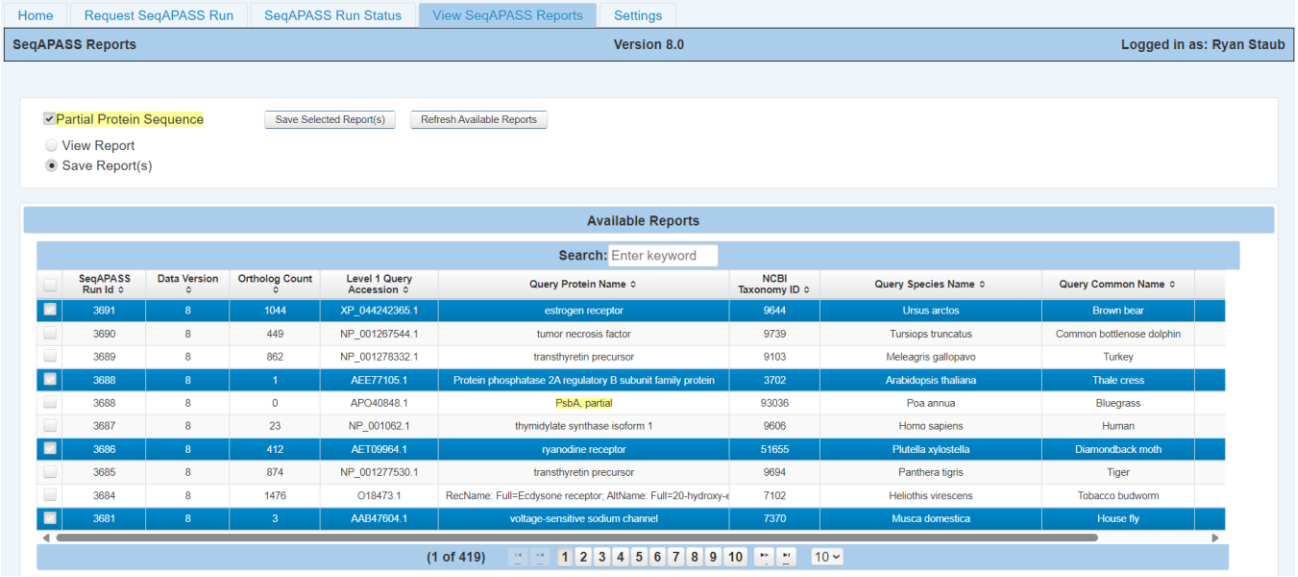
To select a completed run and view Level 1 data, select the corresponding radio button in the first column of the table and click "Request Selected Report." This will open the Level 1 page to view the Level 1 data and to set up queries for Level 2, Level 3, and Level 4.

Note: The user ***MUST*** select a radio button ***PRIOR*** to clicking "Request Selected Report." If the user fails to select a radio button and clicks "Request Selected Report" a Spinning Wheel will appear and disappear, and no completed run will be opened. Further, ***there is no pop-up message*** indicating that the user did not select a radio button.

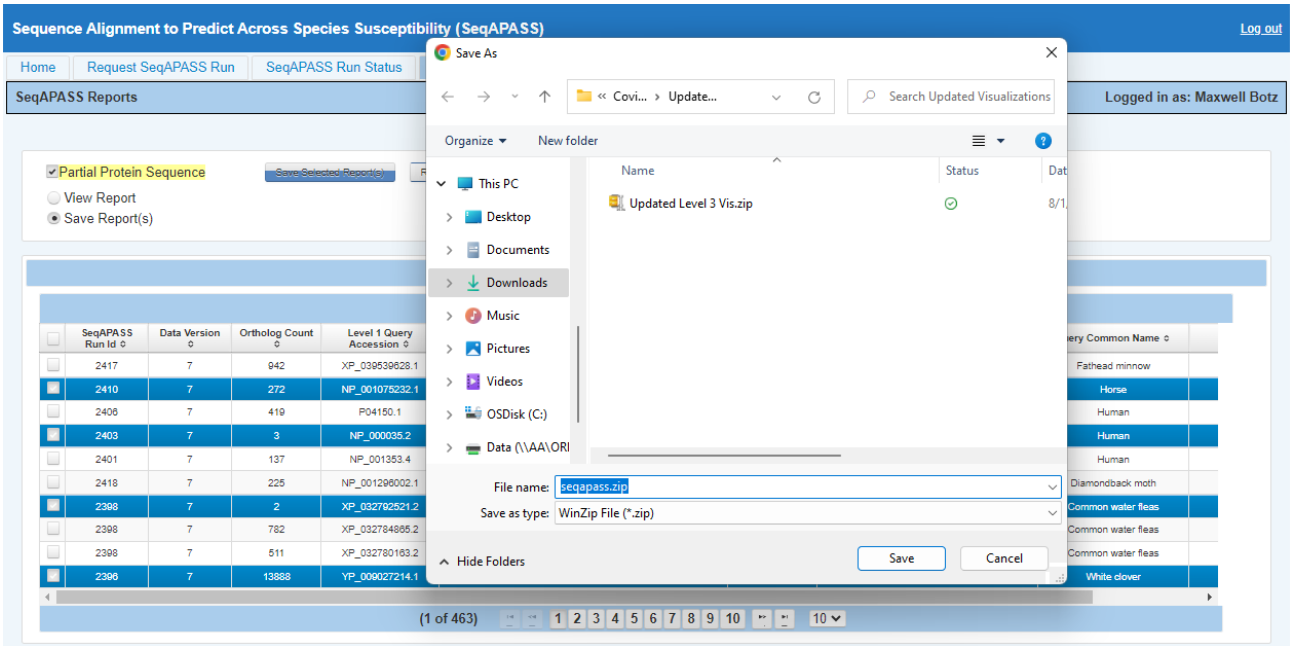


Save Report(s)

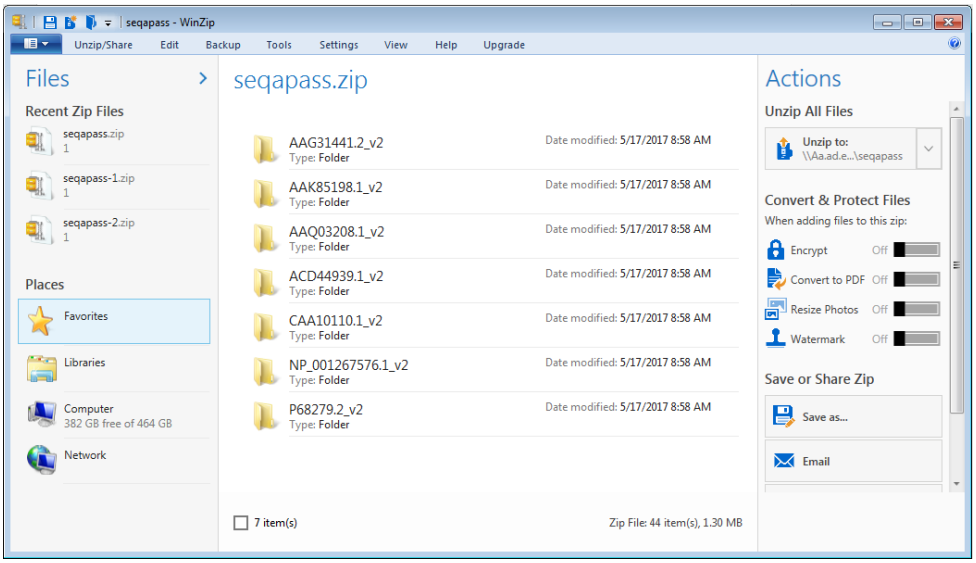
To download completed Level 1, 2, and3 data, select the "Save Report(s)" radio button. Upon doing so the user can select which accession(s) to download by clicking the checkbox in the first column of the table associated with desired accession and click "Save Selected Report(s)."



A WinZip file will be created for all the selected Reports.

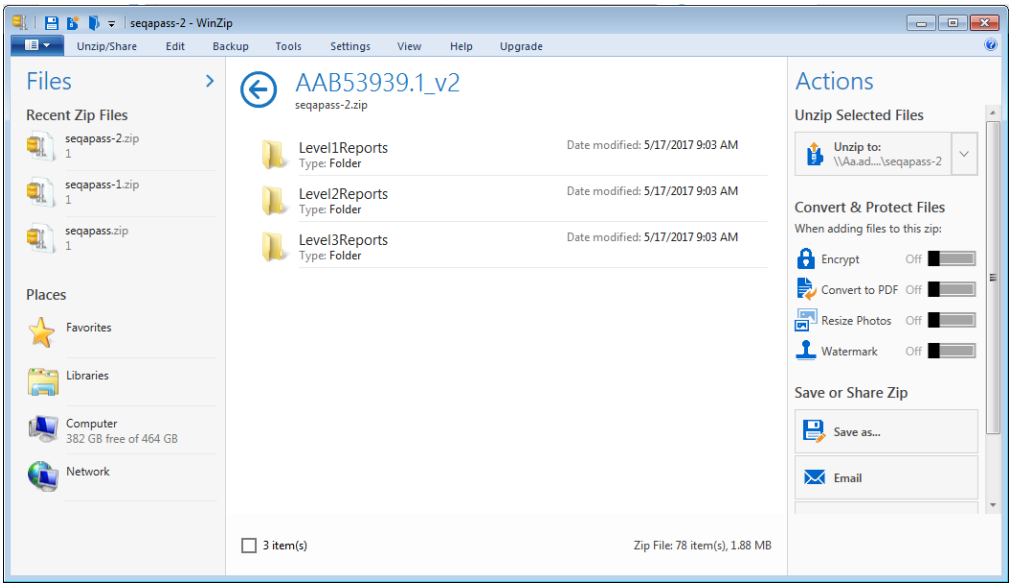


A pop-up seqapass.zip file should appear with data files for each selected report. The naming convention is the NCBI Protein Accession and the Data Version (e.g., AAG31441.2_v6).

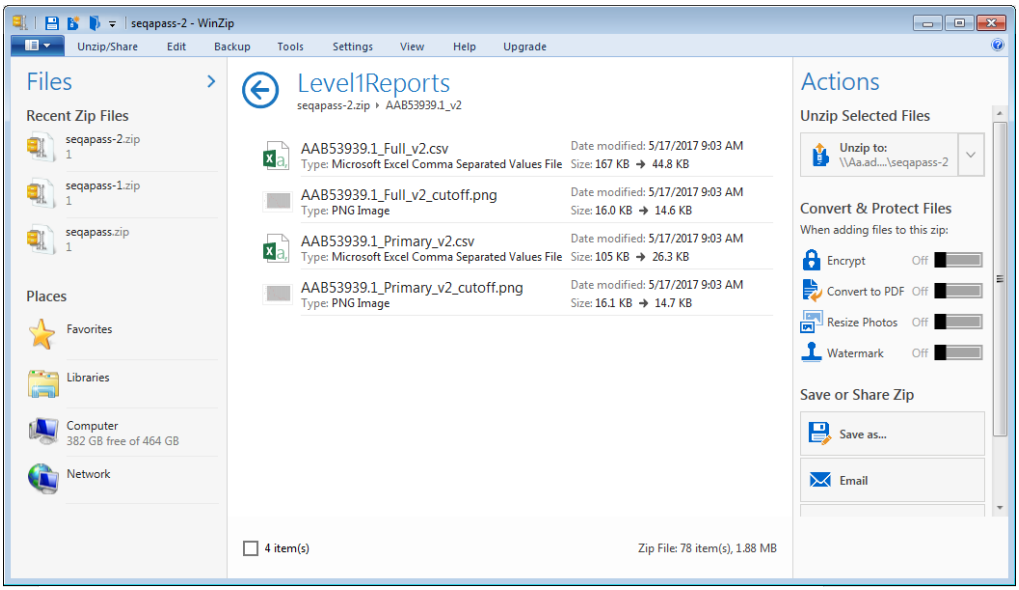


By clicking on one of the Reports for a Protein Accession_version, all available files for each Level of the SeqAPASS evaluation are available.

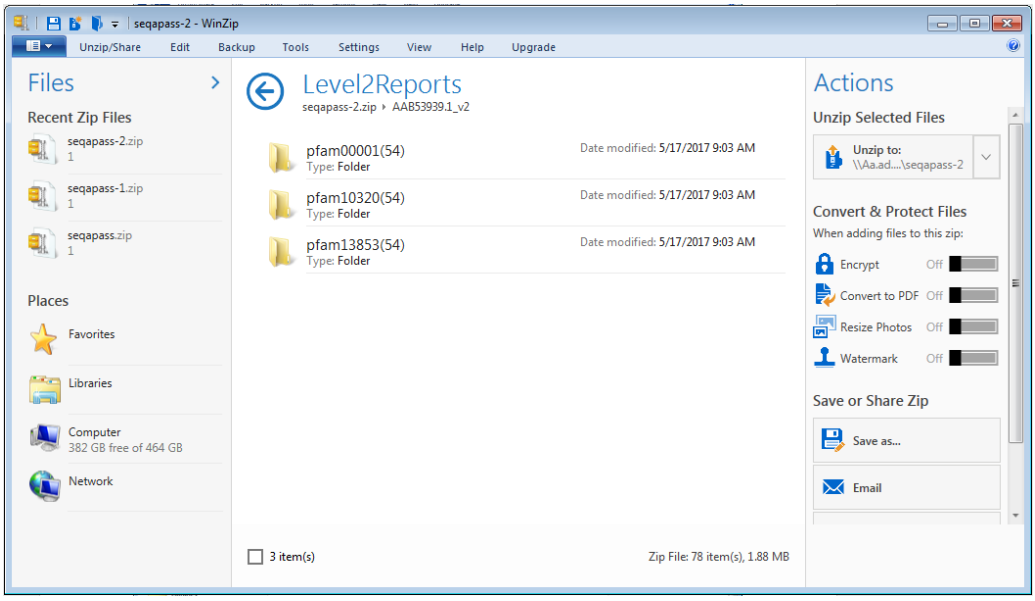
Note: This download includes default settings only. If susceptiblity cut-off or any defaults were manipulated on Level 1 or 2 pages they will ***NOT*** be downloaded here and can ***ONLY*** be downloaded directly from the Level 1 or Level 2 page where the setting was manipulated by the user. Also, data visualizations can ***ONLY*** be downloaded from the Level 1 and 2 pages. They ***DO NOT*** populate in the zip file folders.



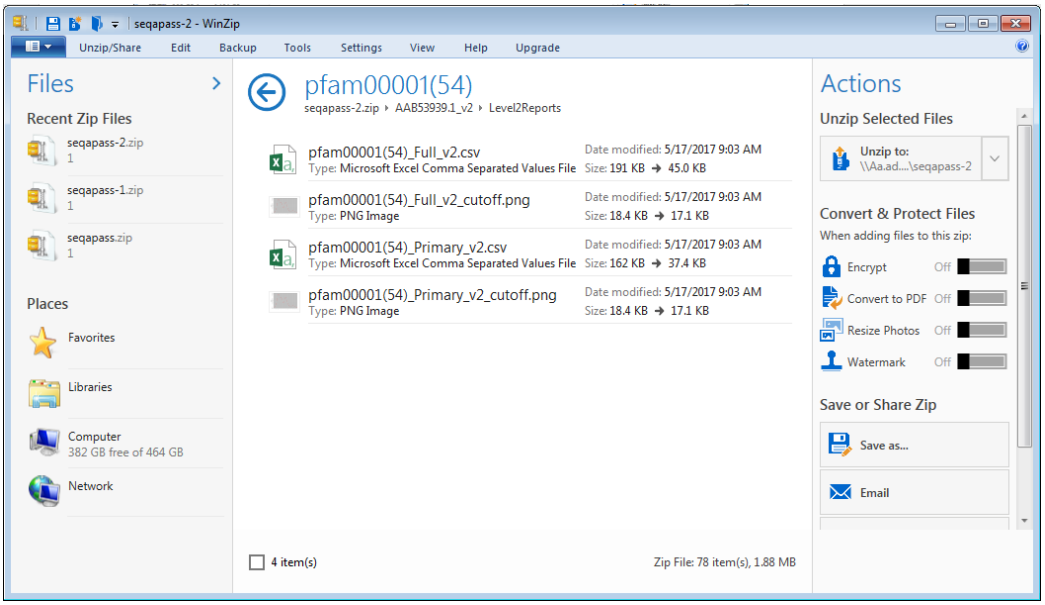
By selecting “Level1Reports”, both full and primary reports are available as csv files as well as a graphic of the density plot for determining the susceptibility cut-off.



By selecting “Level2Reports”, all completed domain comparisons will be available and named by NCBI domain accession with the starting amino acid residue position for the domain (e.g., pfam00001(54)).

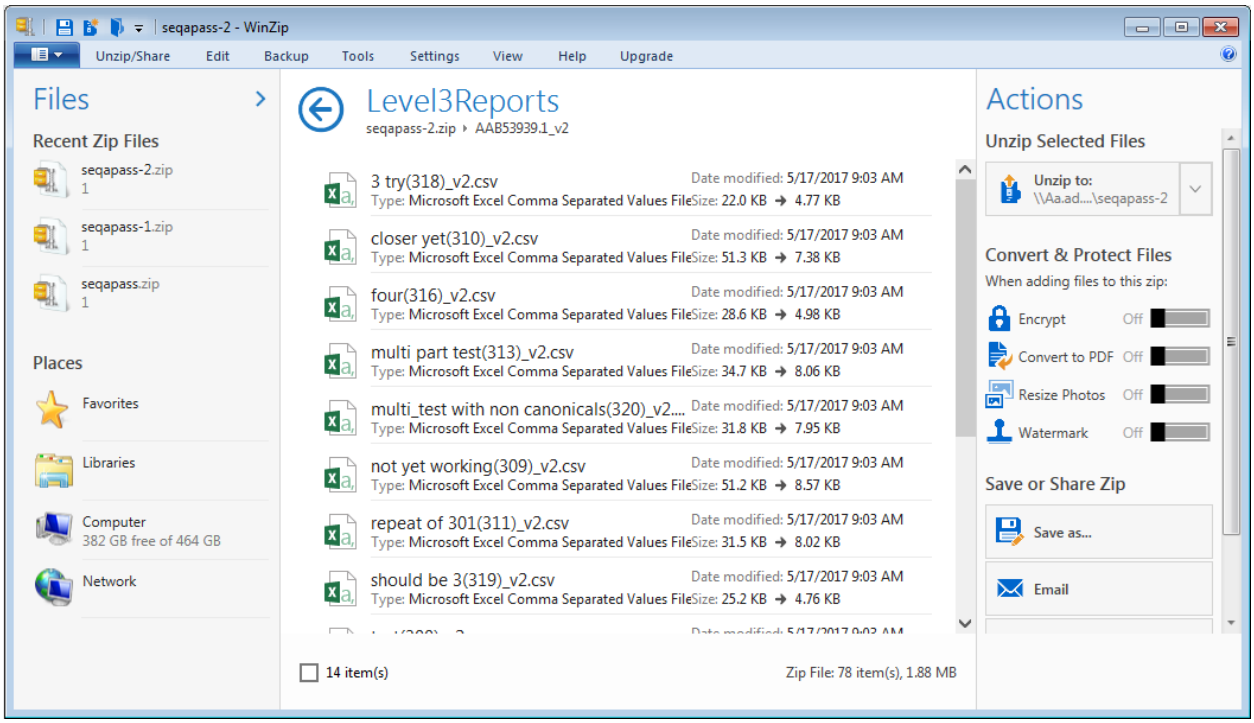


Upon selecting a domain file to view, both full and primary reports are available as csv files as well as a graphic of the density plot for determining the susceptibility cut-off.



By selecting “Level3Reports”, all user defined Level 3 alignments are available as csv.

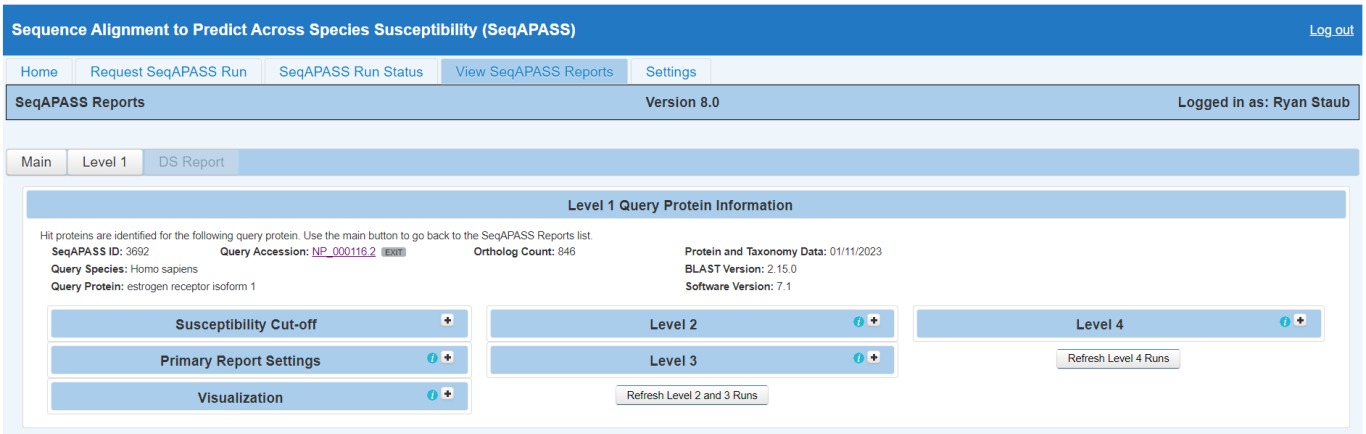
Note: These csv files show the alignments across the entire sequence, not just those amino acid residues selected by the user.



Level 1: Primary Amino Acid Sequence Alignment

From the "View SeqAPASS Reports" tab, upon selecting a radio button and clicking "Request Selected Report" the Level 1 data will be displayed.

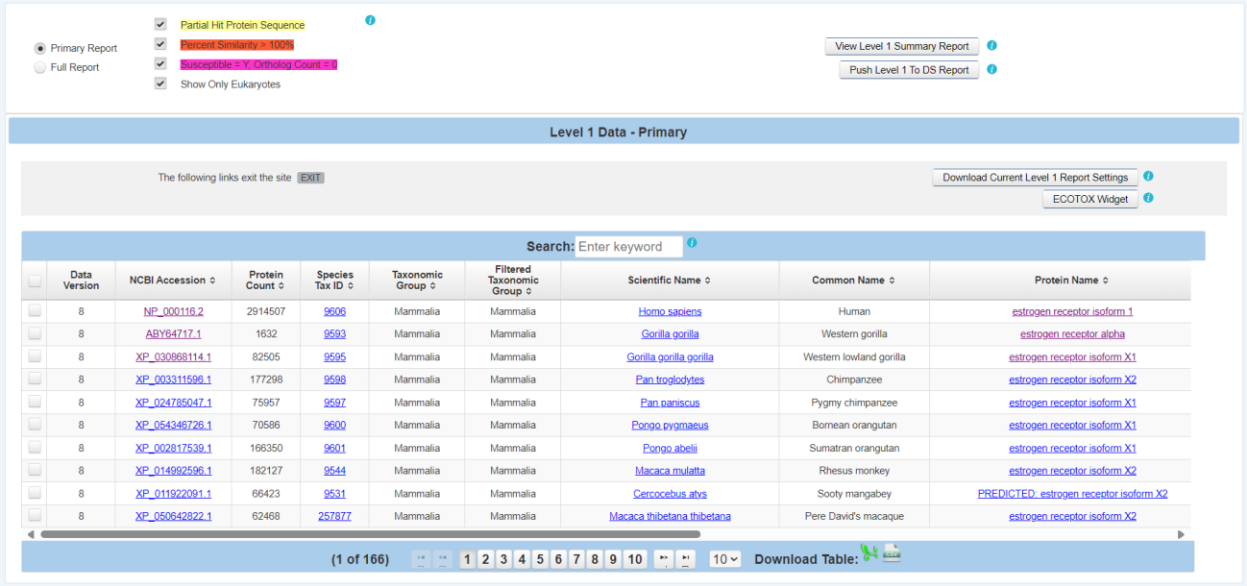
The "Level 1 Query Protein Information" box contains the SeqAPASS Run ID, Query Accession, Ortholog Count (# of hits identified as ortholog candidates to the query species protein sequence), NCBI Data updates ("Protein and Taxonomy Data:" displays the date that NCBI databases were downloaded and incorporated into the SeqAPASS database; "BLAST Version:" and "Software Version:" display the versions being used by the SeqAPASS tool for the selected data), Query Species, and Query Protein. Other information in this box will be described below.



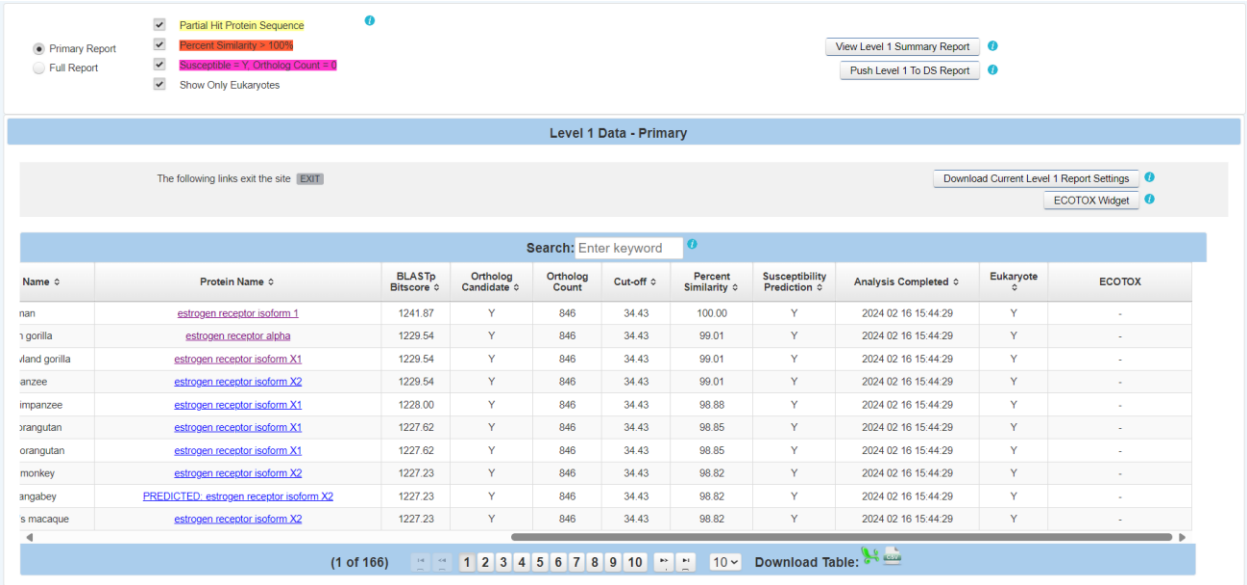
The default table displayed at the bottom of the page is the "Primary Report", which includes query protein information in the first row below the column titles, followed by hit proteins whose sequences aligned with the query protein. The hit proteins are ordered from the highest to lowest percent similarity (Maximum percent similarity =100%). For each hit protein, Data version, NCBI Accession and species information is provided including the "Protein Count" which indicates the number of protein records per species in the NCBI protein database, taxonomic information (See **Primary Report Settings** section below in user guide for more detail on "Taxonomic Group" versus "Filtered Taxonomic Group" columns), and species names. Also included are the NCBI protein accession, protein name, BLASTp bitscore (describes overall quality of the alignment, See NCBI BLASTp tutorials), and percent similarity ([hit bitscore/query bitscore]*100). If the hit protein has been identified as an ortholog candidate (using reciprocal best hit blast method), it will be noted with a "Y" for yes or if not an ortholog candidate, a "N", for no. If the hit protein is predicted to be susceptible according to the susceptibility cut-off criteria, that will also be noted with a "Y" for yes or alternatively an "N" for no. The date the analysis was completed is also identified. The data also includes a column describing the number of ortholog candidates identified using the reciprocal best hit BLAST method. The susceptibility cut-off is also listed in a column. The cut-off is determined through identifying local minimums in the density plot of the percent similarity values for the primary report data set and evaluation of ortholog candidates. Additionally, there is a column that identifies if the species is a Eukaryote noted with a "Y" for yes or alternatively an "N" for no. Links out to the NCBI Protein Database, NCBI Taxonomy Database, and ECOTOX Knowledgebase (specific to the data row) are embedded in the Level 1 data table for "NCBI Accession," "Species Tax ID," "Scientific Name," "Protein Name", and "ECOTOX" columns. (See **Search, View, and Download Data Tables** section of user guide for more information).

Default highlights identify partial protein sequences, sequences with a bitscore higher than the query sequence and therefore percent similarity greater than 100% (commonly synthetic constructs), and when zero ortholog candidates are identified (in this case a user should consider a different query sequence or check the full report). Please see **Susceptibility Cutoff Box for Level 1** section of user guide for details when no orthologs are detected. Additionally, the default setting for the report shows only eukaryote data if a eukaryote is selected as the query protein, excluding prokaryote data from the table with the "Show Only Eukaryotes" checkbox checked. To view prokaryote data, deselect this checkbox. If a prokaryote is selected as the query protein, the default setting will include both eukaryote and prokaryote data and the "Show Only Eukaryotes" checkbox will not be selected. To limit the data to eukaryotes only, the user would check the "Show Only Eukaryotes" checkbox.

Columns in left side of table:



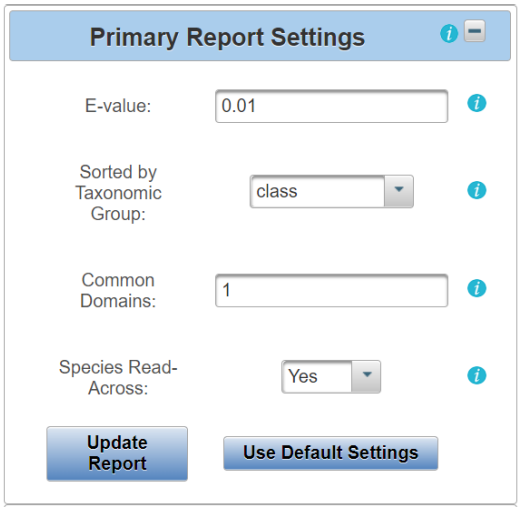
Columns in right side of table:



Level 1: Primary Report Settings

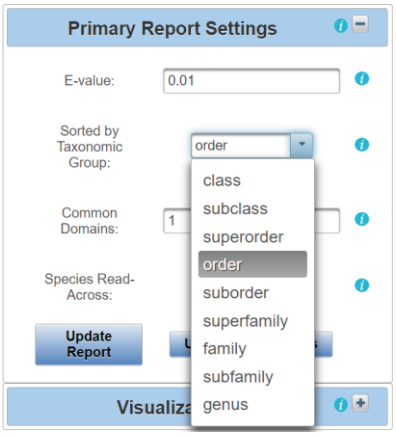
Default settings

The "Primary Report Settings" drop down allows the user to view default settings on the table below and manipulate certain settings. "Primary Report Settings" are only available on the "Primary Report" display, not the "Full Report." The default settings show data for hits whose E-value are ≤ 0.01 and have been identified to have ≥ 1 domain in common with the query sequence. The default setting for the "Sorted by Taxonomic Group" is "class," therefore the "Filtered Taxonomic Group" column in the table is set to identify and report the taxonomic lineage of "class" from the NCBI Taxonomy Database. However, if class is not identified in the NCBI Taxonomic Hierarchy associated with the hit accession, then the algorithm will report the next available Taxonomic Group moving from class to subclass, to superorder, to order, to suborder, to superfamily, to family, to subfamily, to genus. Finally, the susceptibility predictions are set by using species read-across. (Please view **Documentation** Section of the User Guide for details on Read-Across settings). Briefly, Species Read-Across is used to set the susceptibility prediction, where all ortholog candidates are Susceptible = Y; all species listed above the susceptibility cut-off are Susceptible = Y; all species below the cut-off from the same taxonomic group of one or more species above the cut-off are Susceptible = Y; and those below the cut-off that are not ortholog candidates and do not belong to a taxonomic group above the cut-off are Susceptible = N.



Changing Default Settings

The "E-value" and "Common Domains" settings can be manipulated by the user by entering the desired E-value or number of Common Domains in the respective text boxes and clicking "Update Report." The table and data visualization will automatically be updated after a few seconds. The user may choose to change the level of the taxonomic hierarchy that is used for the susceptibility prediction. From the "Sorted by Taxonomic Group" dropdown the user may choose to display a different taxonomic group in the "Filtered Taxonomic Group" column of the data table.



If the user chooses "order" for example, the "Filtered Taxonomic Group" column in the data table will report the taxonomic lineage of "order" from the NCBI Taxonomy Database and all species read-across for the susceptibility prediction will be based on order instead of class. The data visualization will also update. As described previously, if order is not identified in the NCBI Taxonomic Hierarchy associated with the hit accession, then the algorithm will report the next available taxonomic group moving from suborder, to superfamily, to family, to subfamily, to genus. Upon selecting the taxonomic group from the dropdown and clicking "Update Report," the Level 1 Data for the Primary Report will update to the selected taxonomic level.

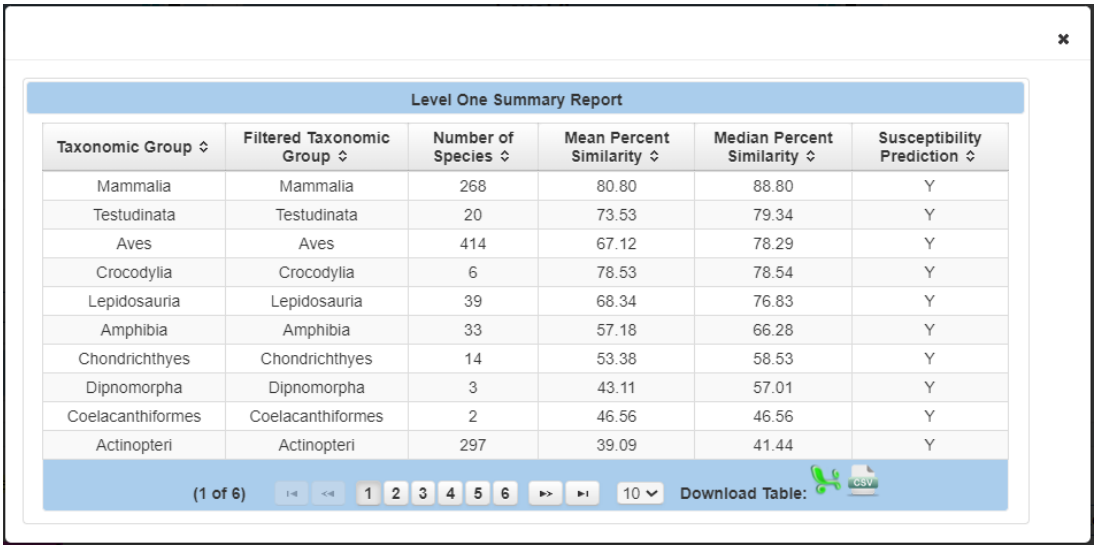
Search: Enter keyword

Data Version	NCBI Accession	Protein Count	Species Tax ID	Taxonomic Group	Filtered Taxonomic Group	Scientific Name	Common Name
8	NP_000116.2	2914507	9606	Mammalia	Primates	Homo sapiens	Human
8	ABY64717.1	1632	9593	Mammalia	Primates	Gorilla gorilla	Western gorilla
8	XP_030868114.1	82505	9595	Mammalia	Primates	Gorilla gorilla gorilla	Western lowland gorilla
8	XP_003311596.1	177298	9598	Mammalia	Primates	Pan troglodytes	Chimpanzee
8	XP_024785047.1	75957	9597	Mammalia	Primates	Pan paniscus	Pygmy chimpanzee
8	XP_054346726.1	70586	9600	Mammalia	Primates	Pongo pygmaeus	Bornean orangutan
8	XP_002817539.1	166350	9601	Mammalia	Primates	Pongo abelii	Sumatran orangutan
8	XP_014992596.1	182127	9544	Mammalia	Primates	Macaca mulatta	Rhesus monkey
8	XP_011922091.1	66423	9531	Mammalia	Primates	Cercocebus atys	Sooty mangabey
8	XP_050642822.1	62468	257877	Mammalia	Primates	Macaca thibetana thibetana	Pere David's macaque

(1 of 166) 1 2 3 4 5 6 7 8 9 10 10 Download Table:

Level One Summary Report

The user can view a summary of the data for each taxonomic group by clicking on the "View Level 1 Summary Report" button. The data includes, number of species, mean percent similarity, median percent similarity and susceptibility prediction. This data can also be downloaded.

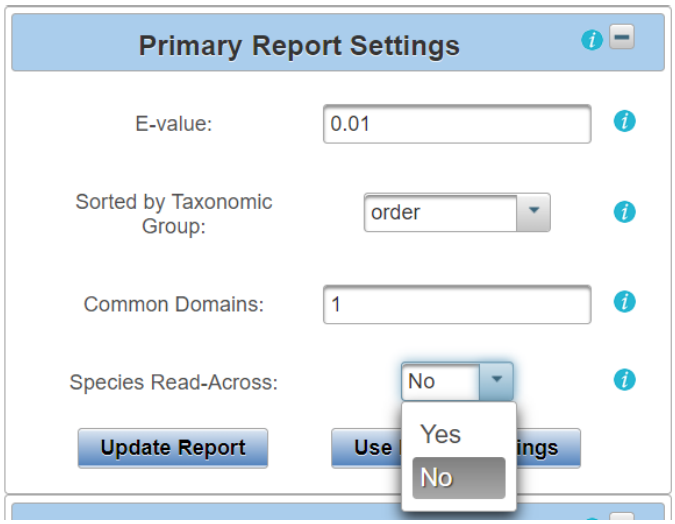


Level One Summary Report

Taxonomic Group	Filtered Taxonomic Group	Number of Species	Mean Percent Similarity	Median Percent Similarity	Susceptibility Prediction
Mammalia	Mammalia	268	80.80	88.80	Y
Testudinata	Testudinata	20	73.53	79.34	Y
Aves	Aves	414	67.12	78.29	Y
Crocodylia	Crocodylia	6	78.53	78.54	Y
Lepidosauria	Lepidosauria	39	68.34	76.83	Y
Amphibia	Amphibia	33	57.18	66.28	Y
Chondrichthyes	Chondrichthyes	14	53.38	58.53	Y
Dipnomorpha	Dipnomorpha	3	43.11	57.01	Y
Coelacanthiformes	Coelacanthiformes	2	46.56	46.56	Y
Actinopteri	Actinopteri	297	39.09	41.44	Y

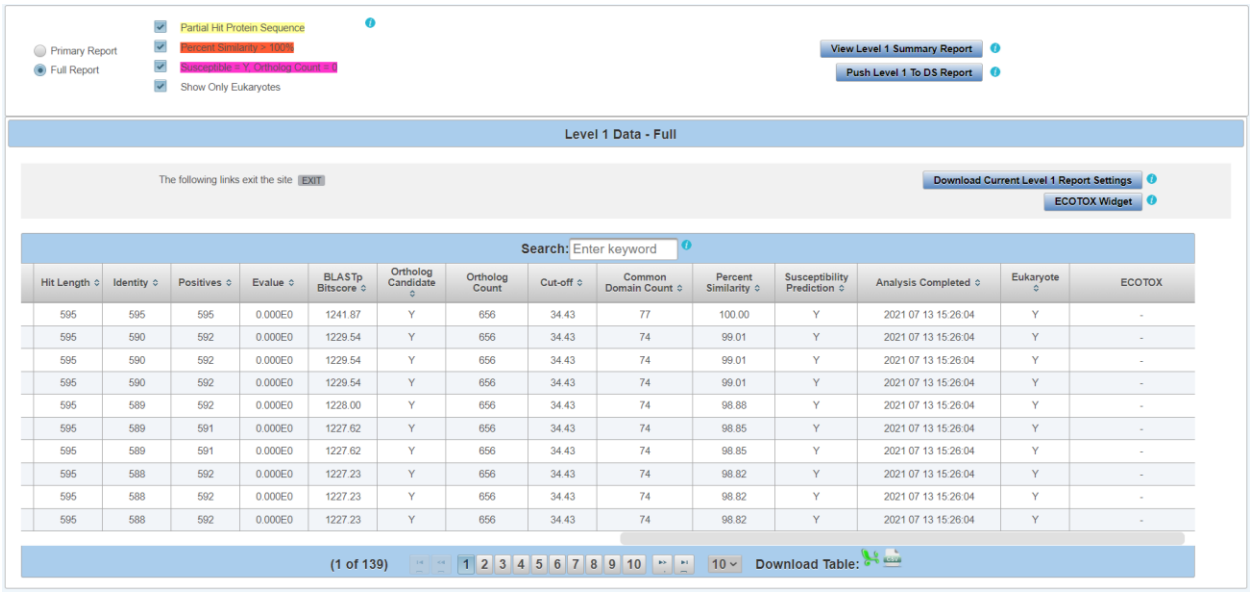
(1 of 6) 1 2 3 4 5 6 10 Download Table:

The user may also choose to turn species read-across off, by using the "Species Read-Across" drop-down and selecting "No" and clicking "Update Report." When "No" is selected, the susceptibility predictions will only be "Y" in the table below if Percent Similarity is above the Cut-off or if the hit is identified as an Ortholog Candidate, yes or "Y." Any hit below the cut-off will yield a susceptibility prediction of no or "N."



The user can select the "Full Report" on the "Level 1" page, which includes the same information as the "Primary Report" and additional information pertaining to the alignment of the protein sequence using BLASTp. Additional information includes the number of amino acid residues in the sequence (Hit Length), the number of exact matching amino acids between the hit and query sequence (Identity), the number of exact and similar matches in amino acids between the hit and the query sequence (Positives), the expect value (E-value) describing the number of different alignments expected to occur in the database search by chance, and the conserved domain count. The conserved domain count identifies all domains associated with the query protein in the NCBI conserved domains database (Specific hits, Non-specific hits, Superfamilies, and Multi-domains; See NCBI conserved domains database for details). SeqAPASS algorithms record the query sequence coverage of each curated domain and compares that coverage to that of the hit sequence. If the hit sequence covers the curated domain greater than or equal to

the query sequence, then the domain is considered a common domain between the hit and query. The number of common domains comparing each hit sequence to the query sequence are summed and reported. This column displays "0" when the hit protein and query protein do not have any common domains. (See **Search, View, and Download Data Tables** section of user guide for more information). The user can also download the currently applied report settings by selecting the "Download Current Level 1 Report Settings." This csv allows the user to track which settings were used or changed by the user when downloading a data table.



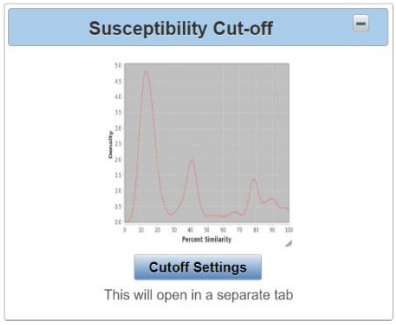
Note: SeqAPASS v2.0 and newer parse the BLASTp query and hit accessions to identify all the species/accessions from identical proteins. Therefore, if a hit sequence represents multiple species, all species with the identical sequence will be found in the data tables for Level 1. To determine which sequence/species was identified from BLASTp as a hit and which sequence/species was parsed from the identical sequence, view the "Full Report" for Level, column "Identical Protein," where "N" is indicative of the original hit sequence and "Y" is the parsed sequence.

	A	B
1	Level 1 Report Settings	
2		
3		
4	Analysis TimeStamp	2019 05 16 11:04:08
5	SeqAPASS version	3.2
6	Query Species	Homo sapiens
7	Query Protein	estrogen receptor isoform 1
8	Query Accession	NP_000116.2
9	Ortholog Count	348
10	L1 Cutoff	Default
11	L1 Cutoff Value	33.93221513
12	E-value	0.01
13	Sorted by Taxonomic Group	CLASS
14	Common Domains	1
15	Species Read Across	Y
16	Show Only Eukaryotes	Checked
17	Report	Primary

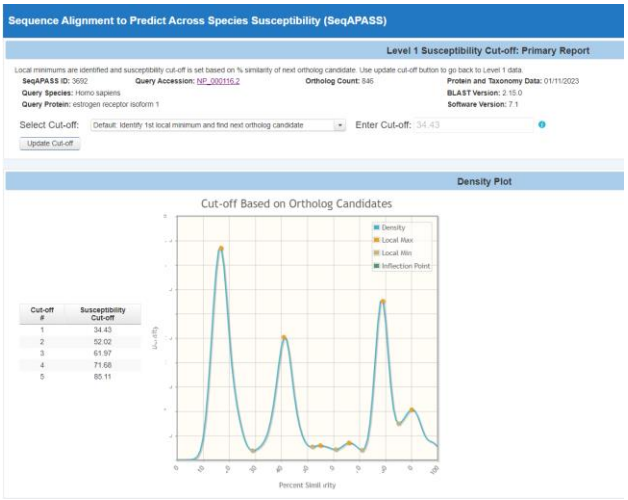
When downloading the current Level 1 report settings, the following information will be present in the csv file. If the user decides to change the default settings, the csv file can be utilized for quick information if the SeqAPASS page is no longer open.

Susceptibility Cutoff Box for Level 1

The susceptibility prediction is determined by identifying ortholog candidates, sequences above a defined susceptibility cutoff, or by identifying those species below the susceptibility cut-off from an organism class above the susceptibility cutoff. The default susceptibility cut-off is set by plotting the distribution of percent similarities calculated for each hit protein. From this plot, the critical points are identified, and the local minimums and maximums reported. Using the ortholog candidate data, a susceptibility cut-off is automatically determined by identifying the first ortholog candidate at an equal or higher percent similarity than the first local minimum. The user can view this graph by clicking the "Cutoff Settings" button in the "Susceptibility Cut-off" box, which will open a new tab in the web browser. The "Select Cut-Off" drop-down can allow the user to select between the default cut-off, the 2nd local minimum or a user defined cut-off. The 2nd susceptibility cut-off is identified in the density plot by finding the 1st ortholog candidate at an equal or higher percent similarity to that of the 2nd local minimum. Upon selecting the User defined cut-off from the dropdown, the user can view and closely examine the density plot and manipulate the cut-off. The "Enter Cut-off" text box becomes active and the user can enter a number 1-100. To update the cut-off in the Level 1 data report and/or close the cutoff tab and return to the Level 1 page, click "Update Cut-off" button.

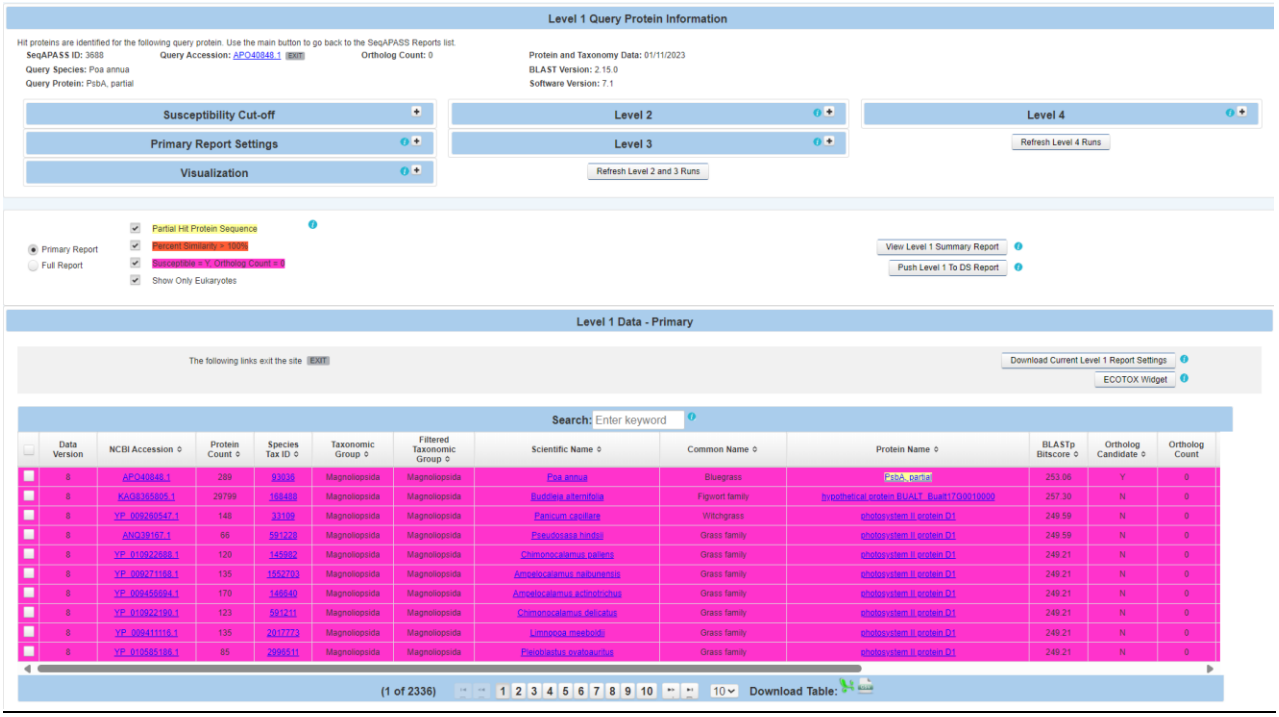


Note: The user should have a justification for changing the susceptibility cut-off, either based on evaluation of Ortholog cutoffs in the data visualization or from empirical evidence.

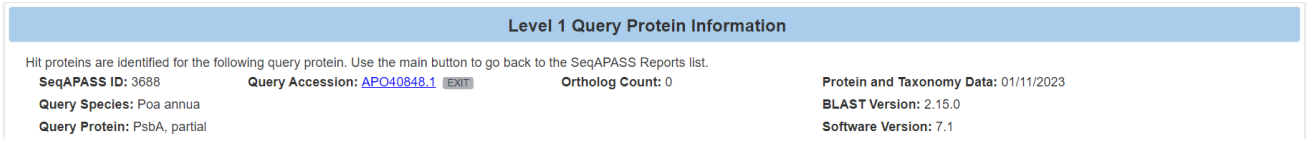


All potential susceptibility cut-offs generated by the data distribution and ortholog candidate identification are reported in the table with columns "Cut-off #" and "Susceptibility Cut-off". The user can use these numbers to define a cut-off if empirical evidence suggests that the "Default" or "2nd minimum" are not supported.

No Orthologs Detected



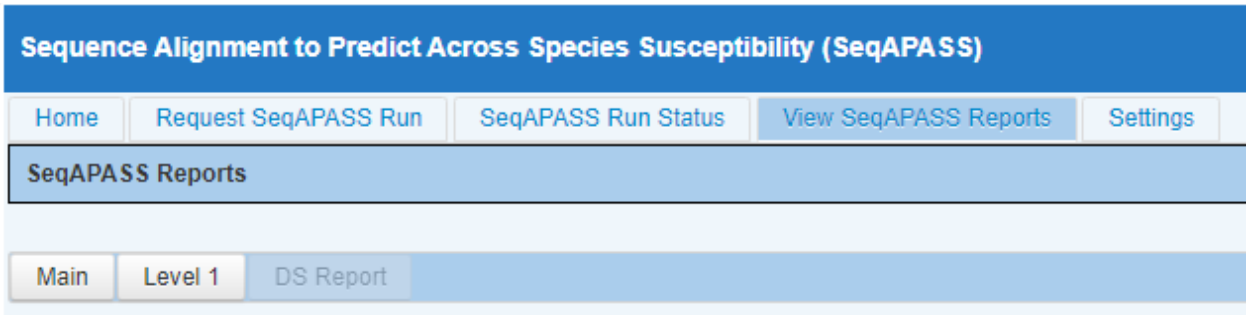
If no orthologs are detected from reciprocal best hit blast analysis, the "Ortholog Count" will be "0" at the top of the "Level 1 Query Protein Information" page. The cutoff will be set by the local minimums only, therefore the susceptibility prediction will NOT consider ortholog candidates. ***It is recommended that the user checks the full report for ortholog candidates or identifies a different query sequence for the susceptibility predictions***. Here, the susceptibility predictions will be highlighted in dark pink in the Level 1 data table to indicate that 0 orthologs were detected and the susceptibility cutoff was determined from plotting the distribution of percent similarities and identifying the local minimums.



Note: De-select the "Show Only Eukaryotes" checkbox to see if prokaryotes were identified as orthologs.

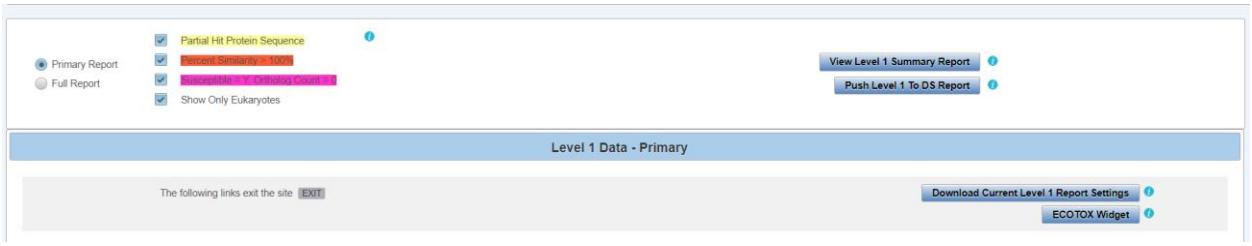
By clicking on the "Cutoff Settings" button when no orthologs are detected, the "Cut-off #" and "Susceptibility Cut-off" columns will report only the local minimum values.

From the "Level 1" page the user can return to the list of completed SeqAPASS runs by clicking the "Main" button on the upper left-hand side of the "Level 1 Query Protein Information" page.



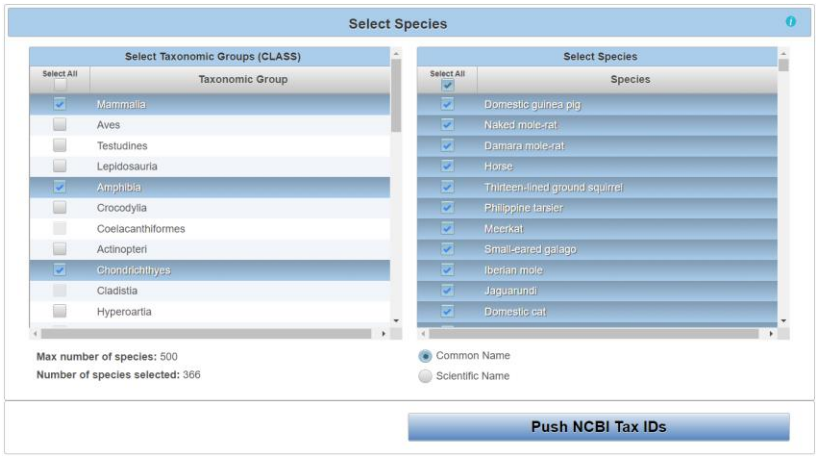
ECOTOX Widget

The ECOTOX widget gives the user the option to create a species and chemical filter that will link out to ECOTOX. The widget allows for rapid access of curated empirical toxicity data from the ECOTOXicology (ECOTOX) Knowledgebase (https://cfpub.epa.gov/ecotox/) that can be compared to sequence-based predictions of chemical susceptibility from SeqAPASS results. In the "Level 1 Data" table header, the "ECOTOX Widget" button can be clicked and will open a widget that is populated with all the taxonomic groups and species from the Level 1 Data table.



Select Species

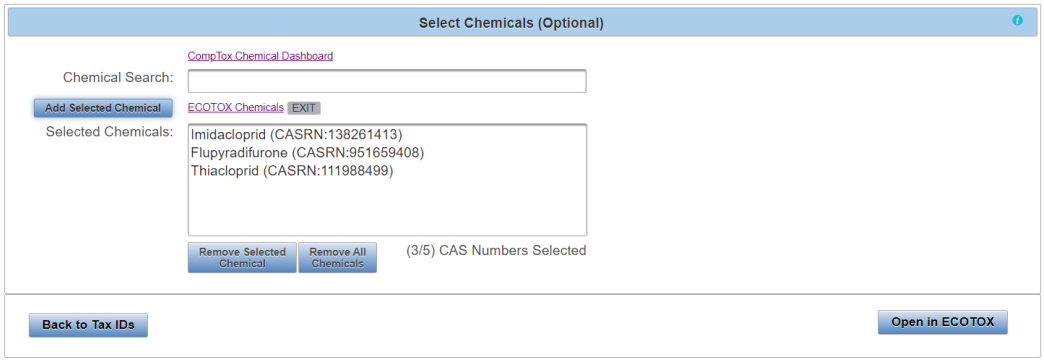
Taxonomic groups that are present within the "Select Species" section of the ECOTOX widget are those found in the Level 1 Data table and Boxplot. Default settings auto select those taxonomic groups and species in common with ECOTOX. The user can select/deselect taxonomic groups of interest in the "Select Taxonomic Groups (CLASS)" box. Additionally, species can be selected/deselected in the "Select Species" box. Taxonomic groups and Species whose selection box is displayed greyed out, are not found in ECOTOX. The maximum number of species that can be pushed to the ECOTOX filter is 500. (Note: common species include those that are in the ECOTOX database, which does NOT mean they have toxicity records associated with them in ECOTOX.). Upon selecting species for comparison in ECOTOX, the user clicks on the "Push NCBI Tax IDs" button to advance to the "Select Chemicals" feature of the widget.



Select Chemicals (Optional)

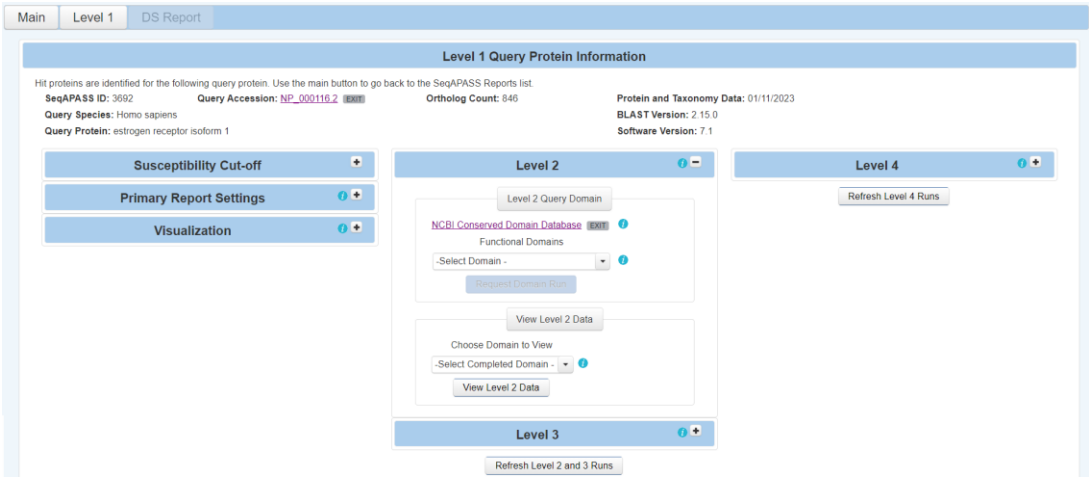
The "Select Chemicals" feature of the ECOTOX widget is optional and can skipped by selecting the "Open in ECOTOX" button. Chemicals can be searched by typing 3 letters of the chemical name which will then populate with the top 100 hits containing those 3 letters. The chemical will appear and display the CASRN number following the name. Up to 5 chemicals can be included in the ECOTOX filter. Unwanted chemicals can be individually removed by selecting chemical and then clicking the "Remove Selected Chemicals" or all chemicals can be removed by clicking the "Remove All Chemicals." There are links to the "CompTox Chemical Dashboard" and "ECOTOX Chemicals," which open the respective databases in separate browser tabs to aid in finding chemicals of interest. To push the created filtered group to the ECOTOX Explore page, click the "Open in ECOTOX" button. Clicking the button will open up a separate browser tab that will incorporate the user customized group within the ECOTOX webpage.

The selected species will be added to an ECOTOX Custom Species Group with the selected chemicals used as a filter in Explore for the user to view and download records from ECOTOX.



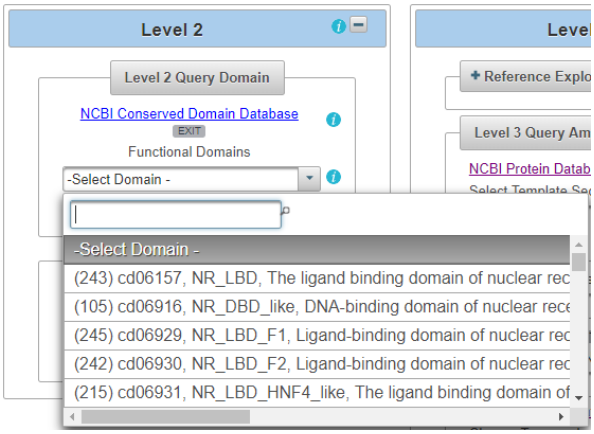
Level 2: Functional Domain(s) Alignment

In the "View SeqAPASS Reports" tab, on the "Level 1 Query Protein Information" page, there is a "Level 2" box for comparing hit domains to the query domain. In the "Level 2" dropdown box, there is a link out to the "NCBI Conserved Domain Database" for the query protein of interest. Below this link the user will find a drop-down containing functional domains associated with the query sequence for comparison across species.



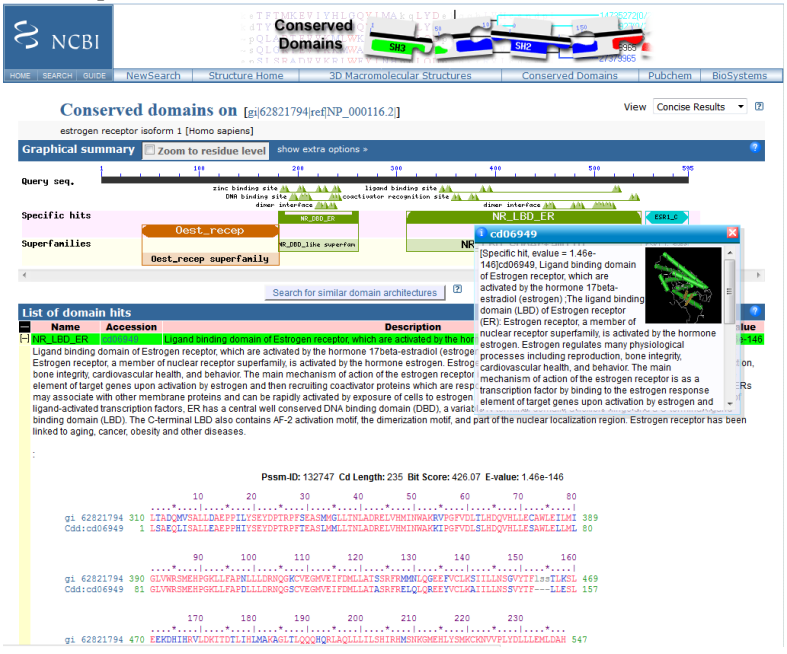
In the drop-down box (below the words “Functional Domains”) the user will find all domains associated with the query protein listed in the “NCBI Conserved Domains Database”. To compare a domain from the query protein to domains of the hit proteins, the user will use the drop-down to highlight a domain and click the “Request Domain Run” button.

Note: Domains in the drop-down are listed with the first amino acid residue position that aligns with the NCBI curated domain in parenthesis, followed by the NCBI domain Accession, domain name, and description.

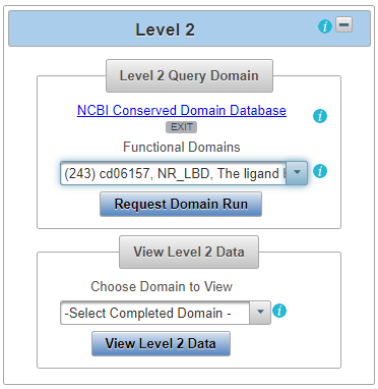


Note: The user can also use the text box on the top of the drop-down to search the “Functional Domain” list in the drop-down.

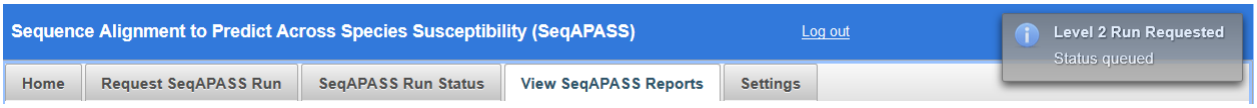
It is recommended that the user click on the “NCBI Conserved Domains Database” http://www.ncbi.nlm.nih.gov/cdd/ link to identify which domains are “Specific hits” in the NCBI Conserved Domains Database. On the NCBI page, the user can scroll over the graphical representation of the domains associated with the query sequence to highlight and identify the Accession associated with domain “Specific hits.” The example below shows the user hovering over the NR_LBD_ER domain with the computer mouse.



After identifying the domain(s) of interest and the corresponding starting residue and domain Accession, the user can return to the SeqAPASS tool, scroll to the domain of interest in the drop-down. If that domain has not been previously run by the user, the "Request Domain Run" button will become active and the user can click it to submit the domain query.



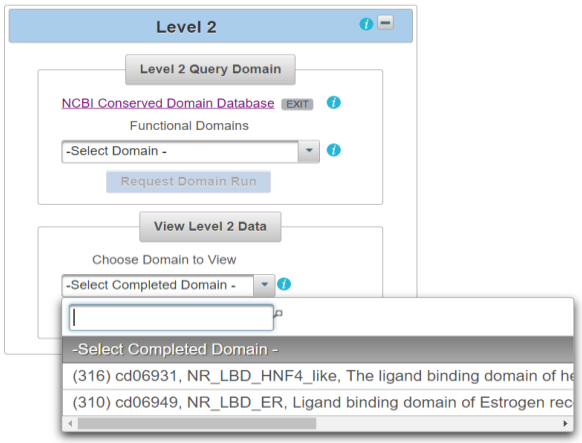
When user clicks the "Request Domain Run" button, the following message will appear if the runs has been submitted successfully.

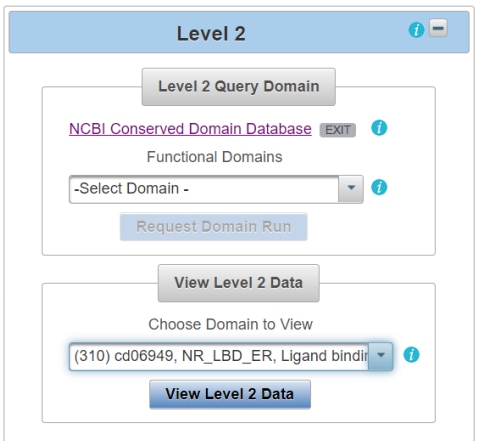


When sequence comparisons have completed for the selected functional domain, the domain will be present in the "View Level 2 Data" drop-down. The ***drop-down is not automatically populated*** with the completed domain run. The ***user must click on the "Refresh Level 2 and 3 runs" button to update the page*** for the newly completed domain to present itself in the Choose Domain to View drop-down.

To view a completed Level 2 domain, highlight the domain of interest in the drop-down box and click the "View Level 2 Data" button. This will bring the user to the "Level 2" data page for the selected query protein/domain.

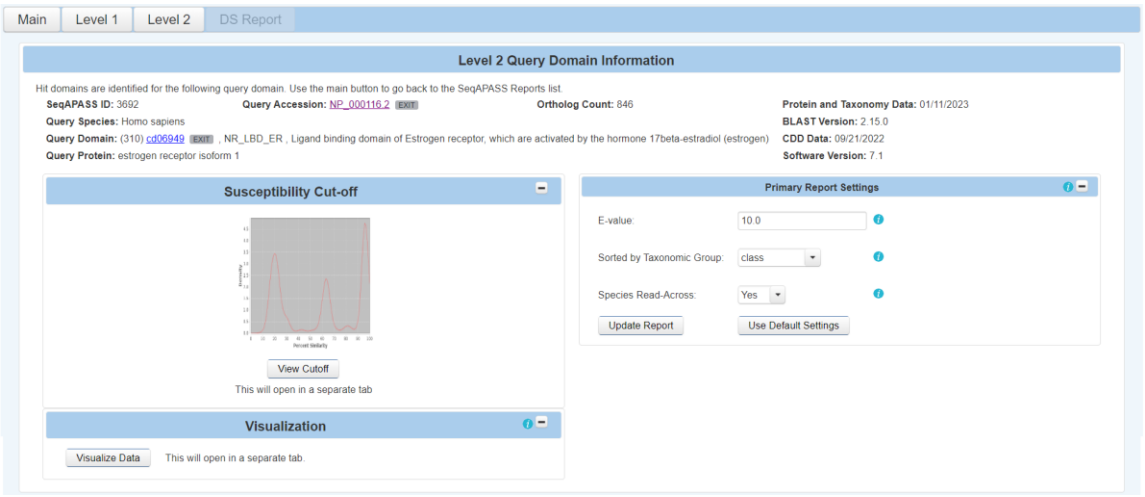
<u>Note:</u> The user can also use the text box on the top of the drop-down to search the "Completed Domain" list.





View Level 2 Data Page

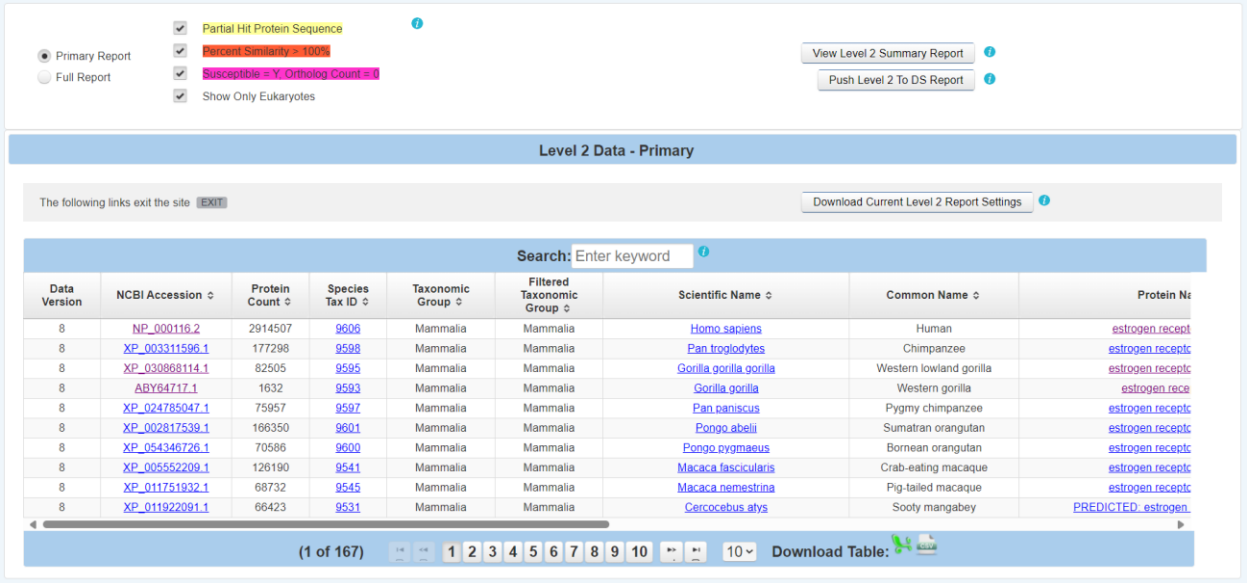
The "Level 2 Query Domain Information" box contains the SeqAPASS Run ID, Query Accession, Ortholog Count (# of hits identified as ortholog candidates to the query species protein sequence), NCBI Data updates ("Protein and Taxonomy Data:" and "CDD Data:" display the dates that NCBI databases were downloaded and incorporated into the SeqAPASS database; "BLAST version:" and "Software Version:" displays the version being used by the SeqAPASS tool for the selected data), Query Species, Query Domain (with link out to NCBI domain page), Query Protein name.



The default "Level 2" table is the "Primary Report", which includes query domain information in the first row below the column titles, followed by hit domains whose sequences aligned with the selected query domain. The hit domains are ordered from the highest to lowest percent similarity (Maximum percent similarity =100%). For each hit domain, Data Version, NCBI Accession and species information is provided, including the "Protein Count" which indicates the number of protein records per species in the NCBI protein database, taxonomic information, and species names. Also included are the NCBI accession for the query protein, query protein name, Domain Type, BLASTp bitscore (describes overall quality of the alignment, See NCBI BLASTp tutorials), and Domain percent similarity ([hit bitscore/query bitscore]*100). If the hit protein has been identified as an ortholog candidate (using reciprocal best hit BLAST method), it will be noted with a "Y" for yes or if not an ortholog candidate, a "N", for no.

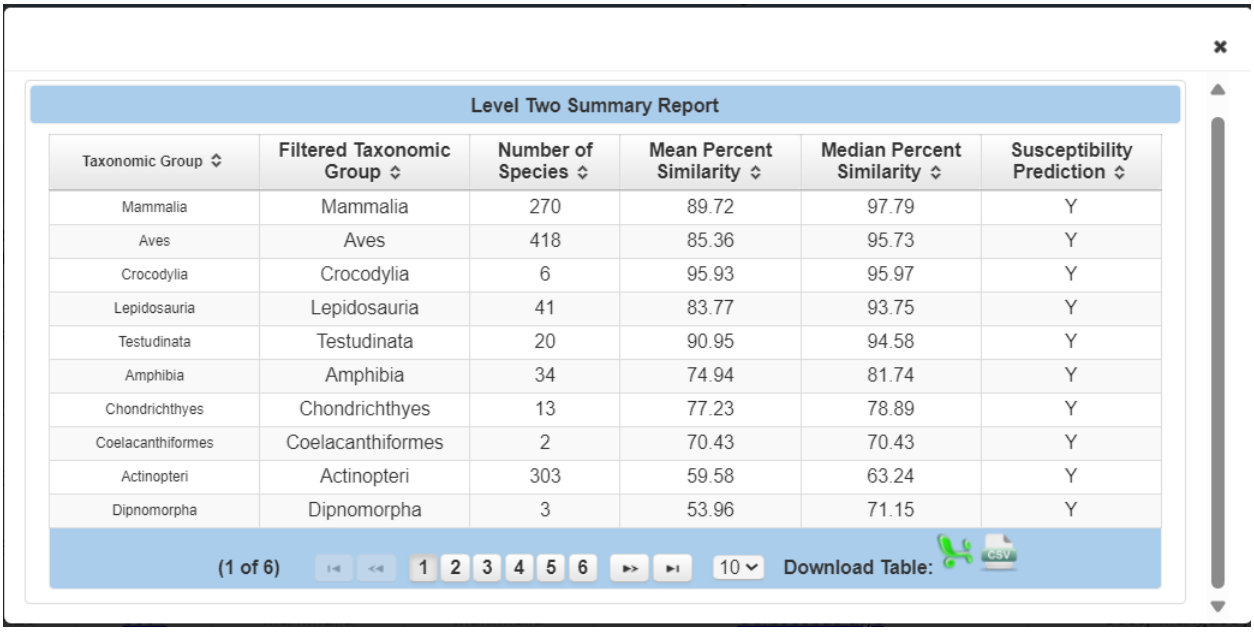
A prediction of susceptibility is displayed based on the susceptibility cut-off, identified with a "Y" for yes or an "N" for no. The date/time the analysis was completed is also identified. (See **Search, View, and Download Data Tables** section of user guide for more information). There is a column that identifies if the species is a eukaryote, noted with a "Y" for yes or alternatively a "N" for no if the hit is a prokaryote. Additionally, a column with a link to the U.S. EPA ECOTOX Knowledgebase (https://cfpub.epa.gov/ecotox/help.cfm) is available when there are empirical toxicity data curated for the species identified in the row. This link allows the user to view available single chemical toxicity data from the literature for specific species.

Default highlights identify partial protein sequences, sequences with a bitscore higher than the query domain and therefore percent similarity greater than 100% (commonly synthetic constructs), and when zero ortholog candidates are identified (in this case a user should consider a different query sequence). Additionally, the default setting for the report shows only eukaryote data, excluding prokaryote data from the table with the "Show Only Eukaryotes" checkbox checked. To view prokaryote data, deselect this checkbox.



Level Two Summary Report

The user can view a summary of the data for each taxonomic group by clicking on the "View Level 2 Summary Report". The data includes, number of species, mean percent similarity, median percent similarity and susceptibility prediction. This data table can also be downloaded.



Level Two Summary Report

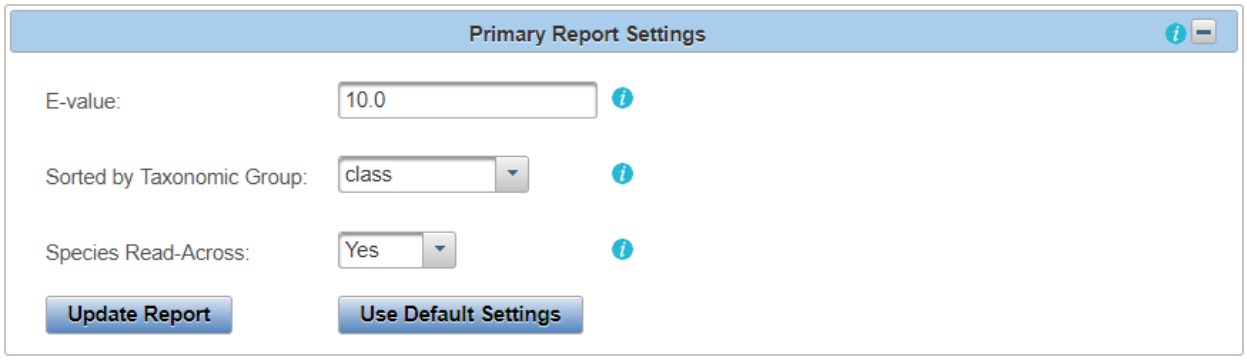
Taxonomic Group	Filtered Taxonomic Group	Number of Species	Mean Percent Similarity	Median Percent Similarity	Susceptibility Prediction
Mammalia	Mammalia	270	89.72	97.79	Y
Aves	Aves	418	85.36	95.73	Y
Crocodylia	Crocodylia	6	95.93	95.97	Y
Lepidosauria	Lepidosauria	41	83.77	93.75	Y
Testudinata	Testudinata	20	90.95	94.58	Y
Amphibia	Amphibia	34	74.94	81.74	Y
Chondrichthyes	Chondrichthyes	13	77.23	78.89	Y
Coelacanthiformes	Coelacanthiformes	2	70.43	70.43	Y
Actinopteri	Actinopteri	303	59.58	63.24	Y
Dipnomorpha	Dipnomorpha	3	53.96	71.15	Y

(1 of 6) 1 2 3 4 5 6 10 Download Table:

Level 2: Primary Report Settings

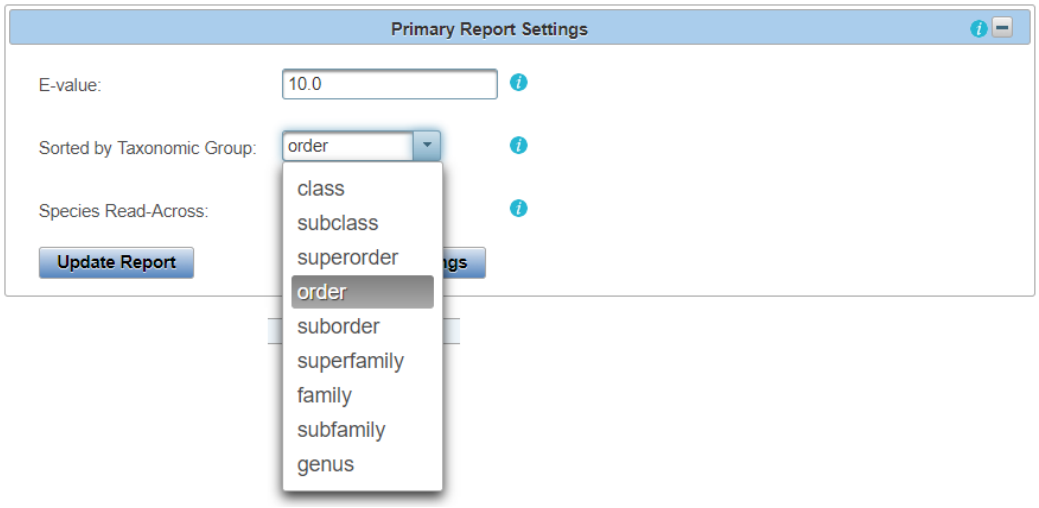
Default settings

The "Primary Report Settings" box allows the user to view default settings on the table below and manipulate certain settings. The "Primary Report Settings" box is only available on the "Primary Report" display. The default settings show data for hits whose E-value are ≤ 10. The default setting for the "Sorted by Taxonomic Group" is "class," therefore the "Filtered Taxonomic Group" column in the table is set to identify and report the taxonomic lineage of "class" from the NCBI Taxonomy Database. However, if class is not identified in the NCBI Taxonomic Hierarchy associated with the hit accession, then the algorithm will report the next available Taxonomic Group moving from class to subclass, to superorder, to order, to suborder, to superfamily, to family, to subfamily, to genus. Finally, the susceptibility predictions are set by using Species Read-Across. (Please view **SeqAPASS Documentation** Section of the User Guide for details on Read-Across settings). Briefly, "Species Read-Across" is used to set the susceptibility prediction, where all ortholog candidates are Susceptible = Y; all species listed above the susceptibility cut-off are Susceptible = Y; all species below the cut-off from the same taxonomic group of one or more species above the cut-off are Susceptible = Y; and those below the cut-off that are not ortholog candidates and do not belong to a taxonomic group above the cut-off are Susceptible = N.

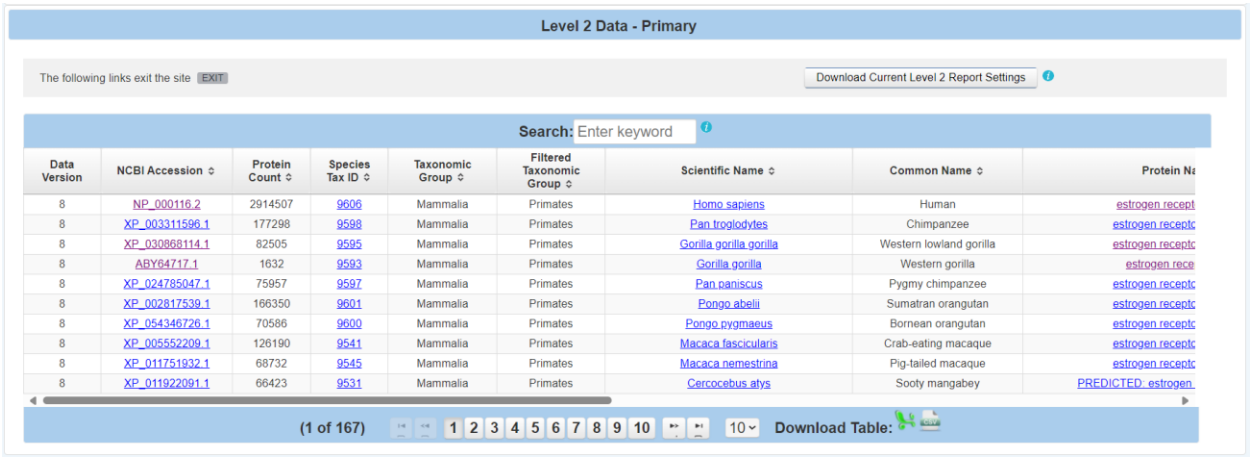


Changing Default Settings

The user may choose to change the level of the taxonomic hierarchy that is used for the susceptibility prediction. From the "Sorted by Taxonomic Group" dropdown the user may choose to display a different taxonomic group in the "Filtered Taxonomic Group" column of the data table.

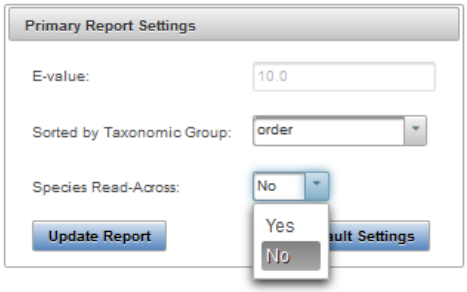


If the user chooses "order" for example, the "Filtered Taxonomic Group" column in the data table will report the taxonomic lineage of "order" from the NCBI Taxonomy Database and all species read-across for the susceptibility prediction will be based on order instead of class. As described previously, if order is not identified in the NCBI Taxonomic Hierarchy associated with the hit accession, then the algorithm will report the next available Taxonomic Group moving from suborder, to superfamily, to family, to subfamily, to genus. Upon selecting the Taxonomic Group from the dropdown and clicking "Update Report," the "Level 2" data for the Primary Report will update to the selected taxonomic level. The user can also download the currently applied report settings by selecting the "Download Current Level 2 Report Settings". This csv file allows the user to track which settings were used or changed by the user when downloading a data table.



Data Version	NCBI Accession	Protein Count	Species Tax ID	Taxonomic Group	Filtered Taxonomic Group	Scientific Name	Common Name	Protein Na
8	NP_000116.2	2914507	9606	Mammalia	Primates	Homo sapiens	Human	estrogen recept
8	XP_003311596.1	177298	9598	Mammalia	Primates	Pan troglodytes	Chimpanzee	estrogen receptc
8	XP_030868114.1	82505	9595	Mammalia	Primates	Gorilla gorilla gorilla	Western lowland gorilla	estrogen receptc
8	ABY64717.1	1632	9593	Mammalia	Primates	Gorilla gorilla	Western gorilla	estrogen rece
8	XP_024785047.1	75957	9597	Mammalia	Primates	Pan paniscus	Pygmy chimpanzee	estrogen receptc
8	XP_002817539.1	166350	9601	Mammalia	Primates	Pongo abelii	Sumatran orangutan	estrogen receptc
8	XP_054346726.1	70586	9600	Mammalia	Primates	Pongo pygmaeus	Bornean orangutan	estrogen receptc
8	XP_005552209.1	126190	9541	Mammalia	Primates	Macaca fascicularis	Crab-eating macaque	estrogen receptc
8	XP_011751932.1	68732	9545	Mammalia	Primates	Macaca nemestrina	Pig-tailed macaque	estrogen receptc
8	XP_011922091.1	66423	9531	Mammalia	Primates	Cercocebus atys	Sooty mangabey	PREDICTED: estrogen

The user may also choose to turn species read across off, by using the "Species Read-Across" drop-down and selecting "No" and clicking "Update Report". When "No" is selected, the susceptibility predictions will only be "Y" in the table below if Percent Similarity is above the Cut-off or if the hit is identified as an Ortholog Candidate, yes or "Y." Any hit below the cut-off will yield a susceptibility prediction of no or "N".



The user can select the "Full Report" on the "Level 2" data page, which includes the same information as the "Primary Report" and additional information pertaining to the alignment of the protein sequence using BLASTp and domain information. Additional information includes the NCBI PSSM ID, NCBI Domain ID, Domain Name, number of amino acid residues in the sequence (Hit Length), the number of exact matching amino acids between the hit and query sequence (Identity), the number of exact and similar (similar side-chain substitutions) matches in amino acids between the hit and the query sequence (Positives), and the expect value (E-value) describing the number of different alignments expected to occur in the database search by chance. (See **Search, View, and Download Data Tables** section of user guide for more information).

Level 2 Data - Full

The following links exit the site EXIT

Download Current Level 2 Report Settings

Search: Enter keyword

Domain Name	Hit Length	Identity	Positive	Evalue	BLASTp Bitscore	Ortholog Candidate	Ortholog Count	Cut-off	Percent Similarity	Susceptibility Prediction	Analysis Completed	Eukaryote	ECOTOX
NR_LBD_ER	238	238	238	2.955E-179	487.26	Y	846	41.50	100.00	Y	2024 02 16 15:48:10	Y	-
NR_LBD_ER	238	237	238	1.806E-178	485.34	Y	846	41.50	99.60	Y	2024 02 16 15:48:10	Y	-
NR_LBD_ER	238	237	238	1.806E-178	485.34	Y	846	41.50	99.60	Y	2024 02 16 15:48:10	Y	-
NR_LBD_ER	238	237	238	1.806E-178	485.34	Y	846	41.50	99.60	Y	2024 02 16 15:48:10	Y	-
NR_LBD_ER	238	237	238	1.806E-178	485.34	Y	846	41.50	99.60	Y	2024 02 16 15:48:10	Y	-
NR_LBD_ER	238	237	238	1.806E-178	485.34	Y	846	41.50	99.60	Y	2024 02 16 15:48:10	Y	-
NR_LBD_ER	238	237	238	1.806E-178	485.34	Y	846	41.50	99.60	Y	2024 02 16 15:48:10	Y	-
NR_LBD_ER	238	237	238	1.806E-178	485.34	Y	846	41.50	99.60	Y	2024 02 16 15:48:10	Y	-
NR_LBD_ER	238	237	238	1.806E-178	485.34	Y	846	41.50	99.60	Y	2024 02 16 15:48:10	Y	-
NR_LBD_ER	238	237	238	1.806E-178	485.34	Y	846	41.50	99.60	Y	2024 02 16 15:48:10	Y	-

(1 of 167) 1 2 3 4 5 6 7 8 9 10 10 Download Table:

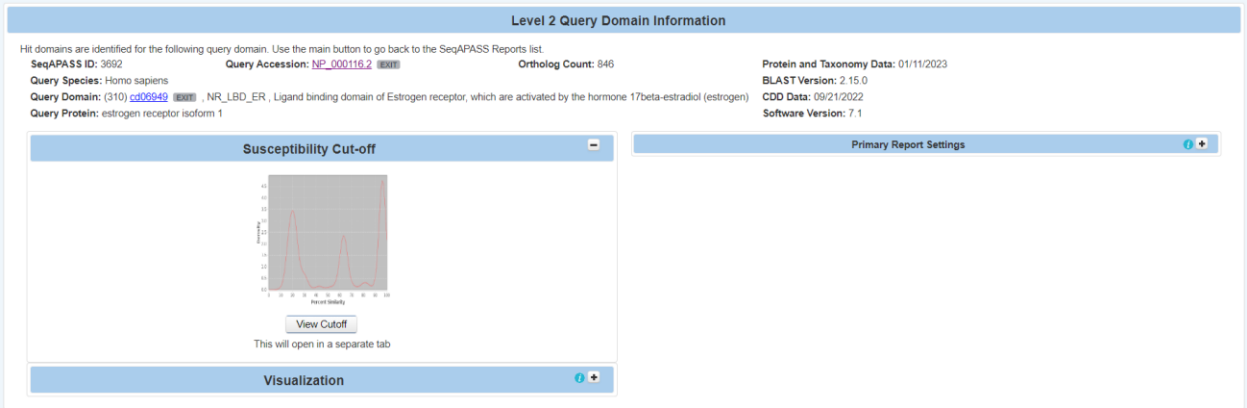
Note: SeqAPASS v2.0 and newer parse the BLASTp query and hit accessions to identify all the species/accessions from identical proteins. Therefore, if a hit sequence represents multiple species, all species with the identical sequence will be found in the data tables for Level 2. To determine which sequence/species was identified from BLASTp as a hit and which sequence/species was parsed from the identical sequence, view the "Full Report" for Level, column "Identical Protein," where "N" is indicative of the original hit sequence and "Y" is the parsed sequence.

	A	B
1	Level 2 Report Settings	
2		
3		
4	Analysis TimeStamp	2019 05 16 11:04:08
5	SeqAPASS version	3.2
6	Query Species	Homo sapiens
7	Query Protein	estrogen receptor isoform 1
8	Query Domain	(310) cd06949, NR_LBD_ER, Ligand binding domain of Estrogen receptor, which are activated by the hormone 17beta-estradiol (estrogen)
9	Query Accession	NP_000116.2
10	Ortholog Count	348
11	L2 Cutoff	Default
12	L2 Cutoff Value	41.5003807
13	E-value	10
14	Sorted by Taxonomic Group	CLASS
15	Species Read Across	Y
16	Show Only Eukaryotes	Checked
17	Report	Primary

When downloading the "Current Level 2 Report Settings", the following information will be present in the csv. If the user decides to change the default settings, the csv can be utilized for quick information if the SeqAPASS page is no longer open.

Susceptibility Cutoff Box for Level 2

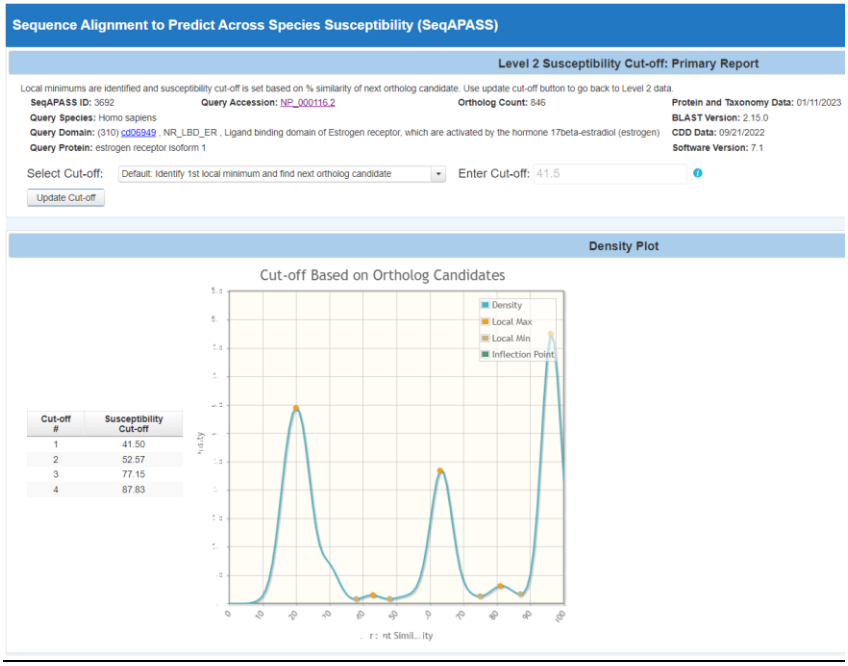
The susceptibility prediction is set by identifying ortholog candidates, sequences above a defined susceptibility cutoff, or by identifying those species below the susceptibility cut-off from an organism class above the susceptibility cutoff. The default susceptibility cut-off is set by plotting the distribution of percent similarities calculated for each hit protein. From this plot, the critical points are identified, and the local minimums and maximums reported. Using the ortholog candidate data, a susceptibility cut-off is automatically determined by identifying the first ortholog candidate at an equal or higher percent similarity than the first local minimum percent similarity. The user can view this graph by clicking the "View Cutoff" button in the "Susceptibility Cut-off" box. Radio buttons located to the right of the graphical display indicate which Cut-off has been applied for the evaluation of susceptibility in the report. These radio buttons can be selected to change the cut-off in the table to the 2nd local minimum, where the 2nd local minimum is identified in the density plot and the first ortholog candidate at an equal or higher percent similarity than the second local minimum percent similarity is used to set the cut-off. Or the user can define the local minimum by clicking on the "User Defined" radio button. Alternatively, the user can view the closely examine the density plot and manipulate the cut-off by clicking the "View Cutoff" button.



Upon clicking "View Cutoff" button, a new page is displayed with a drop-down that allows the user to set the susceptibility cut-off using the first local minimum and the identified ortholog candidate, the second local minimum and the identified ortholog candidate, or by the "User defined cut-off" (where the user selects the cutoff). To update the cut-off in the Level 2 data report and/or return to the Level 2 page, click "Update Cut-off" button.

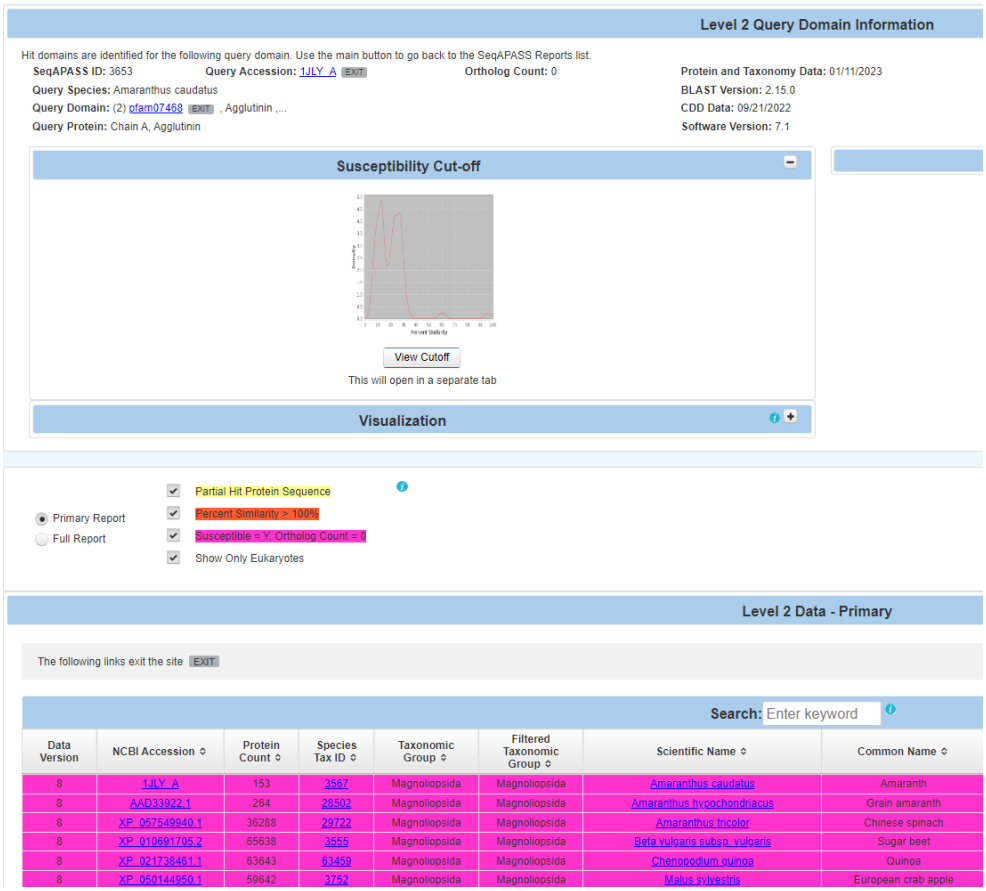
Note: The user should have direct empirical evidence that species above the user defined cutoff are susceptible via the protein of interest, or that the species below the user defined cutoff are not susceptible.

Upon selecting the User defined cut-off from the dropdown, the "Enter Cut-off" text box becomes active and the user can enter a number 1-100.

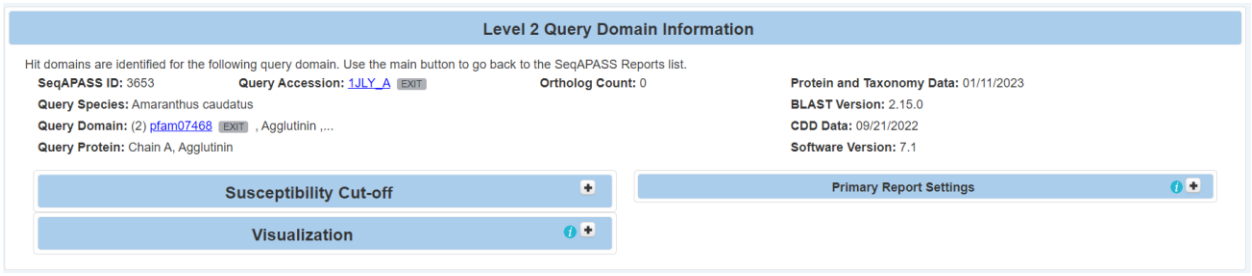


All potential susceptibility cut-offs generated by the data distribution and ortholog candidate identification are reported in the table with columns "Cut-off #" and "Susceptibility Cut-off". The user can use these numbers to define a cut-off if empirical evidence suggests that the "Default" or "2 minimum" are not supported.

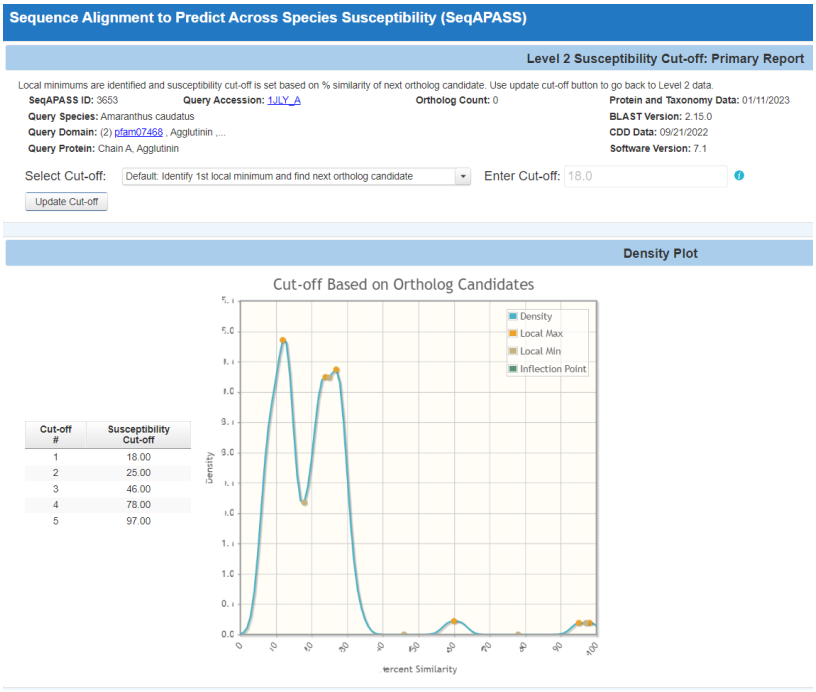
No Orthologs Detected



If no orthologs are detected from reciprocal best hit blast analysis, the "Ortholog Count" will be "0" at the top of the "Level 2 Query Protein Information" page. The cutoff will be set by the local minimums only, therefore the susceptibility prediction will NOT take into account ortholog candidates. ***It is recommended that the user checks the full report for Ortholog candidates or identifies a different query sequence for the susceptibility predictions.*** Here, the susceptibility predictions will be highlighted in dark pink in the Level 2 data table to indicate that 0 orthologs were detected and the susceptibility cutoff was determined from plotting the distribution of percent similarities and identifying the local minimums.



By clicking on the "View Cutoff" button when no orthologs are detected, the "Cut-off #" and "Susceptibility Cut-off" columns will report only the local minimum values.

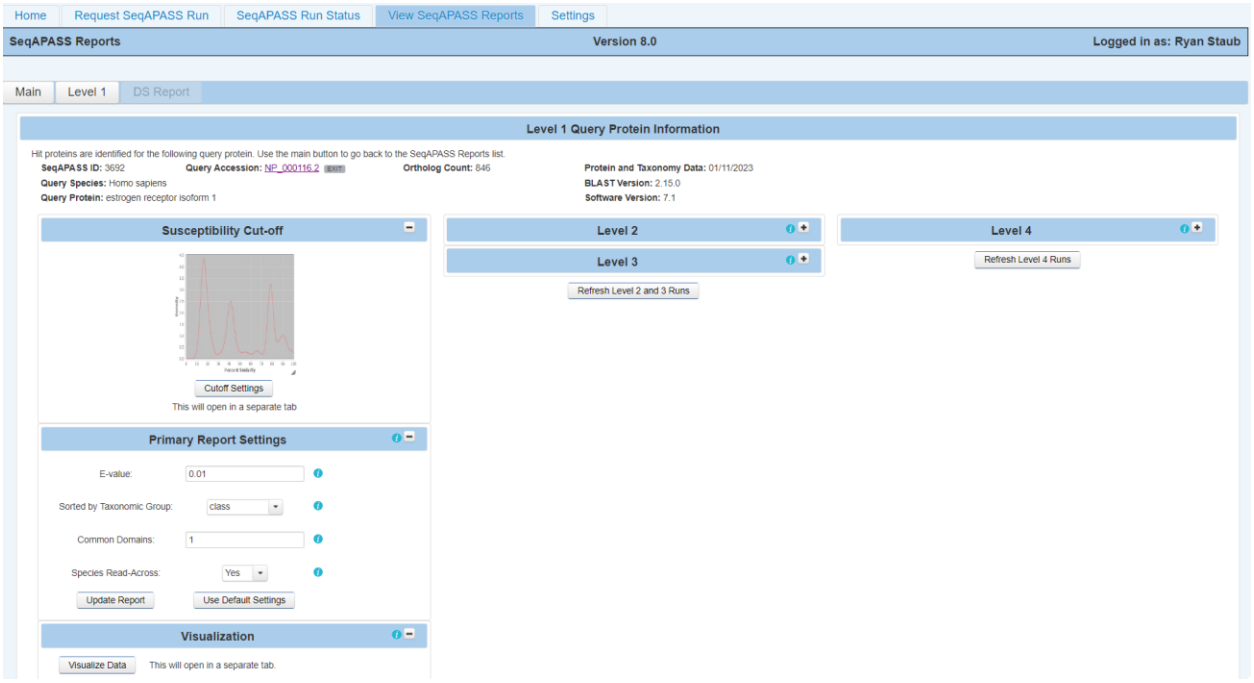


The user can return to the "Level 2" data page by clicking the "Update Cut-off" button or exiting the tab.

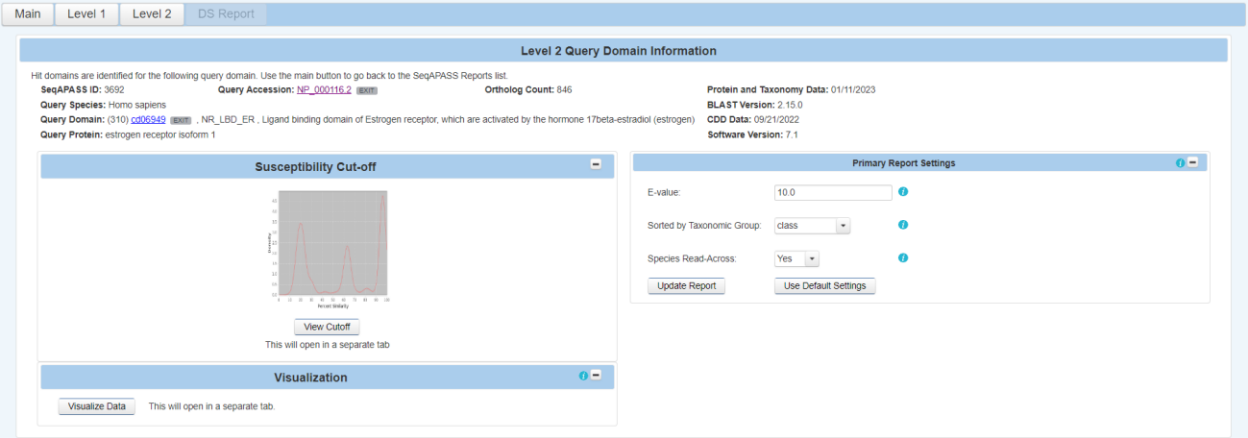
Level 1 and Level 2: Data Visualization

From the Level 1 or Level 2-results page SeqAPASS users can access an interactive data visualization for both the "Primary Report" or "Full Report" by clicking on the "Visualize Data" button.

Example of Level 1 page:



Example of Level 2 page:

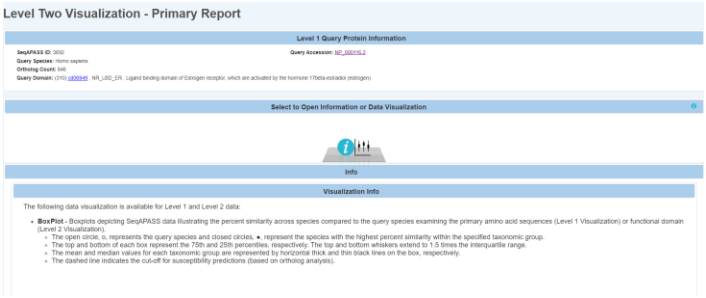


The data visualization will then open in a new web browser tab, one for Level 1 and a different one for Level 2. The visualization will display for the report selected by the user on the Level 1 or Level 2 report page and be identified as “Level One Visualization – Primary Report” or “Level One Visualization – Full Report” and “Level Two Visualization – Primary Report” or “Level Two Visualization – Full Report.”

Note: One report type at a time, either “Primary Report” or “Full Report,” can be displayed in the visualization tab for Level 1 and Level 2. Therefore, if the user is viewing the “Level One Visualization – Primary Report” page and returns to the Level 1 results page and clicks the radio button for “Full Report,” the data visualization tab will update to “Level One Visualization – Full Report.”

Level 1 and 2 Information Page

The initial page that opens upon clicking the “Visualize Data” button provides the respective level query protein information, including SeqAPASS ID, query protein, query species, ortholog count, and query accession information. A link out to the NCBI protein database page corresponding to the queried accession is available by clicking the query accession. Information on the visualization is provided in the “Visualization Info” text box. To view the data visualization boxplots click the BoxPlot icon.

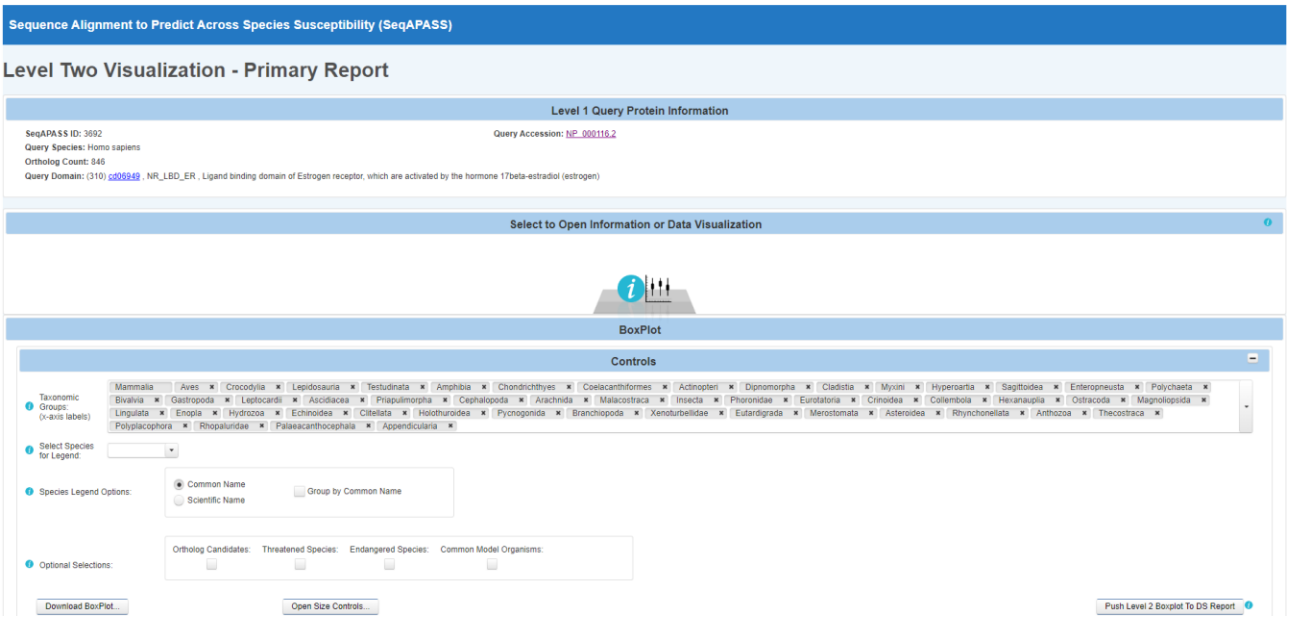


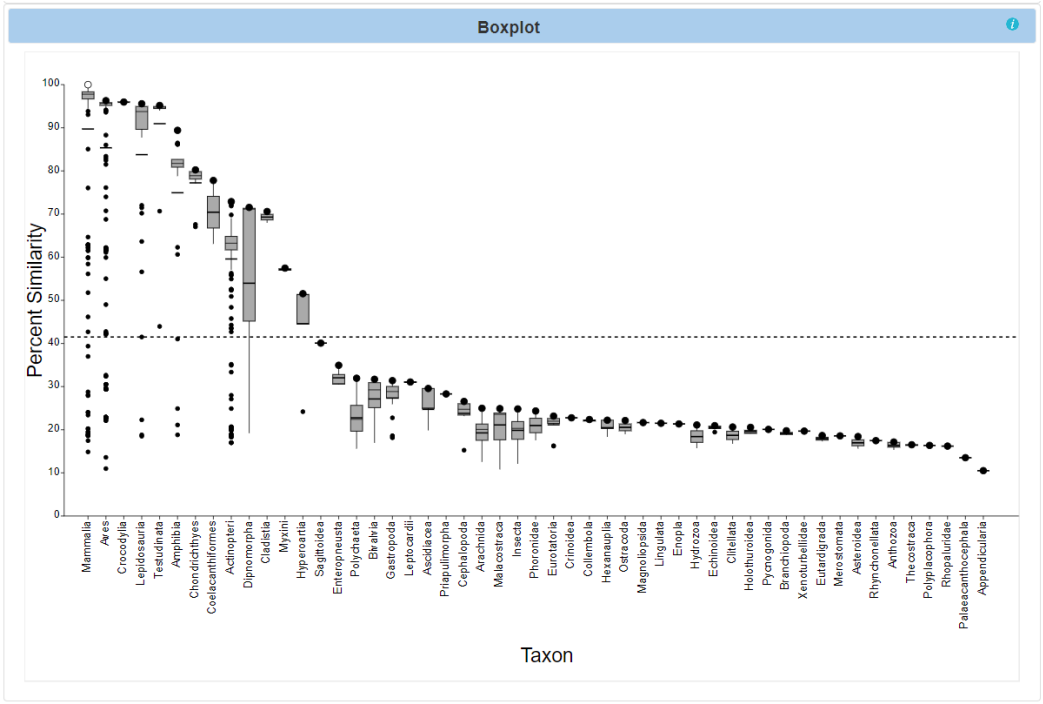
Level 3 Visualization Information Text

- **Heat Map** – Heat Maps depicting SeqAPASS data illustrating the comparison between the template species and the user selected species allow for a summary of species' protein sequence comparisons.
    - The similarity between species compared to the template species and the user selected amino acids is denoted with either a (Y)—yes, or (N)—no. The color green is associated with "yes" and red is associated with "no."
    - Similarities between amino acids are determined by comparing the species-specific amino acids against the template species. The amino acids can be either a Total Match, Partial Match, or Not a Match.
    - The user has the ability to add or remove five settings (Susceptibility Prediction, Susceptibility Prediction Text, Alignment Prediction Heat Map, Amino Acid, and Amino Acid Position) to allow for a customizable Heat Map.
    - Selecting one of the Optional Selections will highlight the species names that are associated with that selection.

Level 1 and 2 BoxPlot Page – Controls

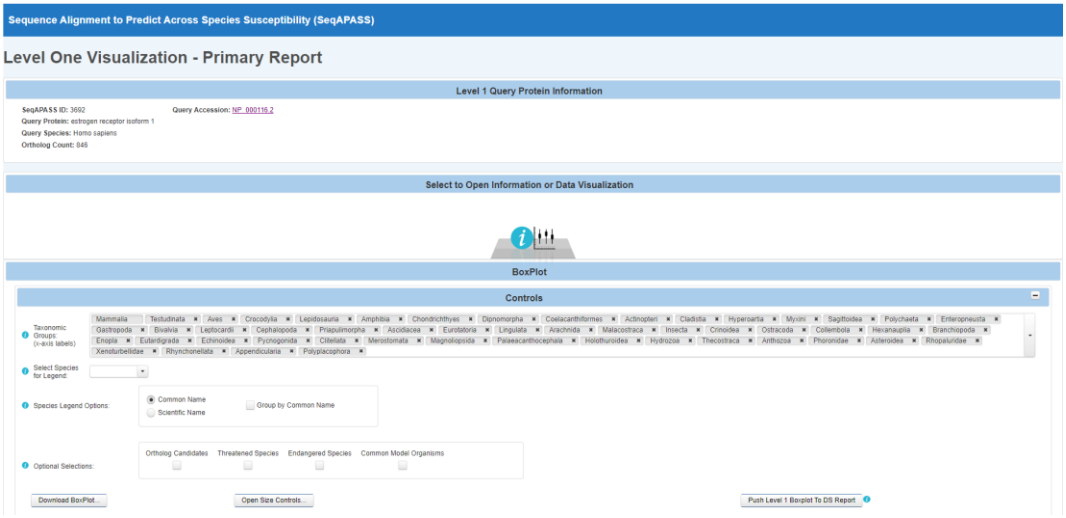
Upon clicking the "BoxPlot" icon on either Level 1 or Level 2 Visualization Information pages, a box for the boxplot "Controls" and a box for the interactive boxplot will open, respectively.

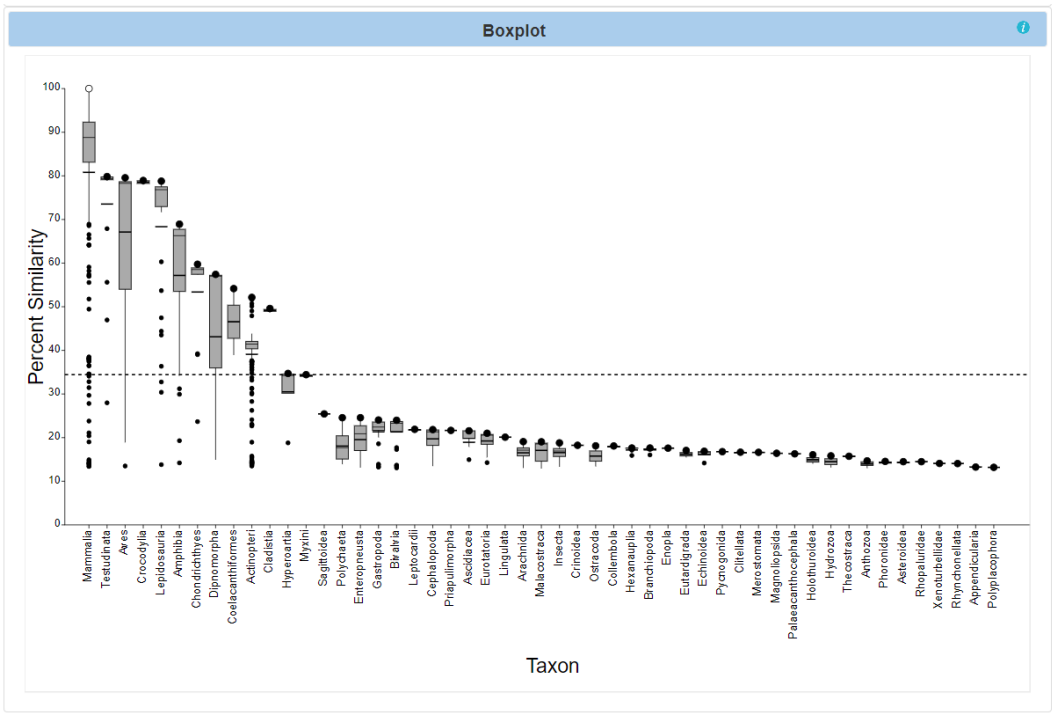




Manipulating Taxonomic Groups on x-axis

The boxplot controls allow the user to edit the taxonomic groups that are displayed on the x-axis by clicking on the "X" for the Taxonomic Group name (e.g., Aves). This action removes the selected group from the x-axis. To the right of the "Taxonomic Groups" controls box is a drop-down that allows the user to remove or add back taxonomic groups to the x-axis of the boxplot graphic, by deselecting or selecting check-boxes in the dropdown. Similarly, unwanted taxonomic groups may be removed directly from the boxplot by hovering the cursor over the taxonomic groups listed along the x-axis. The user will notice that the selection arrow changes to a black arrow with a red 'x' next to it; clicking the taxonomic group will then remove it from the boxplot and the "Taxonomic Groups" controls box. The user can delete multiple species by pressing CTRL and either clicking individual species or slowly dragging across multiple species. Additionally, that taxonomic group will have the checkbox deselected in the "Taxonomic Groups" controls box drop-down list.

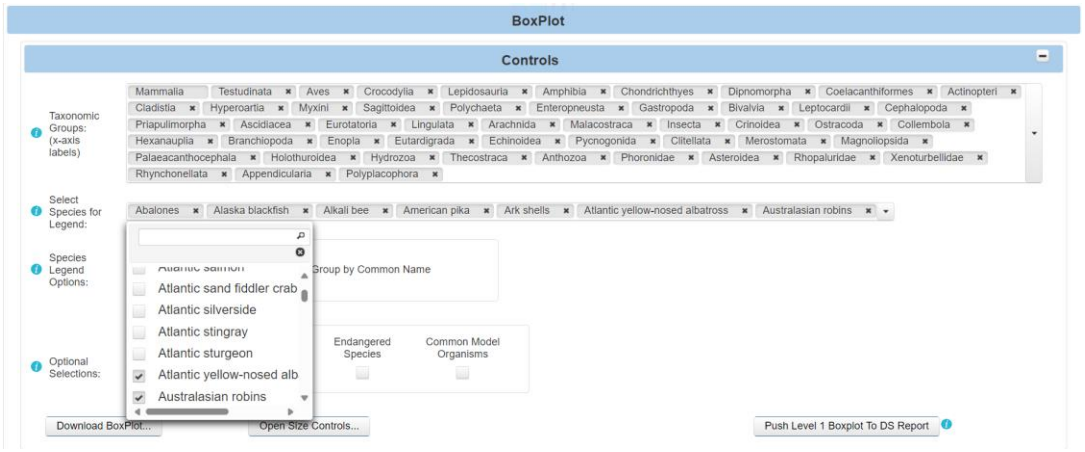


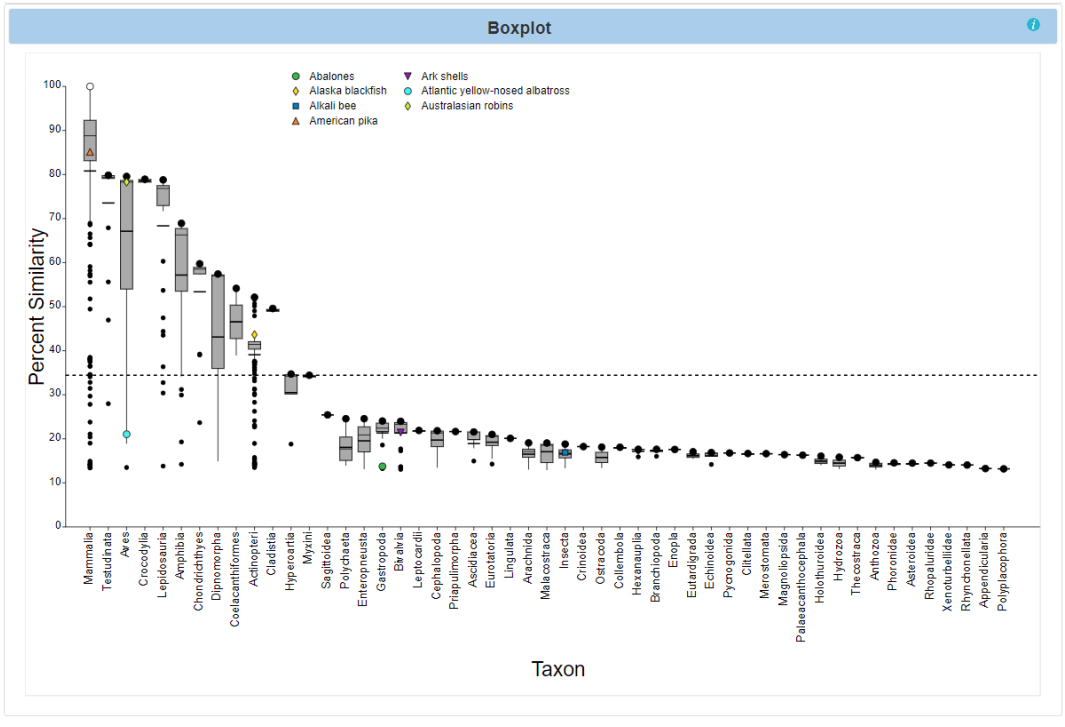


Customize Boxplot Legend

The user may customize the "Boxplot" by adding a legend that will pinpoint species of interest on the boxplot. Upon clicking the drop-down for "Select Species for Legend" in the controls box the user may search in the text box for specific species to display in the boxplot legend. Upon identifying a species from the drop-down menu and selecting the checkbox the species name will be placed in the boxplot legend and a corresponding data point will be produced on the graph. The default settings display the species common name both in the "Select Species for Legend" dropdown and on the boxplot. However, if the species scientific name is desired, the user can select the radio button for "Scientific Name" in the controls box for "Species Legend Options." This action will change the drop-down menu and species in the legend to display the species scientific name.

Note: The database will take a brief moment to update the list upon changing between "Common Name" and "Scientific Name."

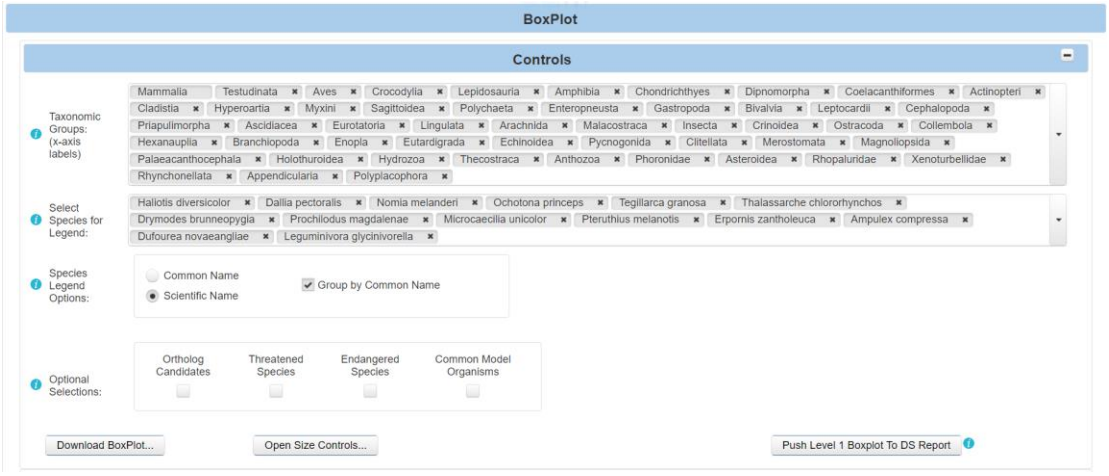


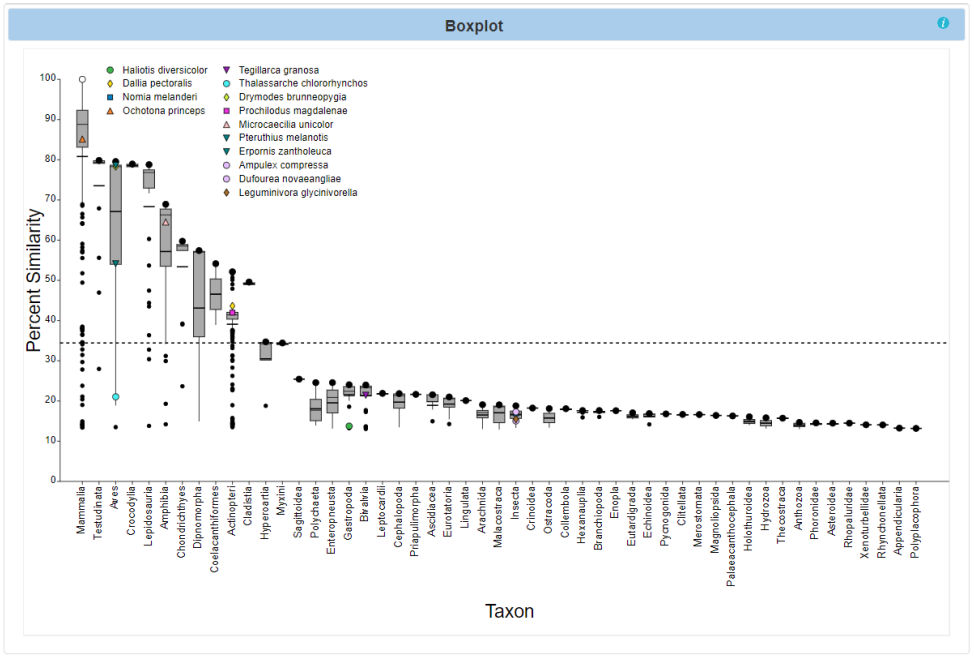


Change Species Display on Plot

Multiple scientific names can be represented by only one common name (e.g., Common name: Babblers; corresponding scientific names: Pteruthius melanotis, Erpornis zantholeuca). Therefore, if a species common name that represents multiple species was used to create the legend, and the user decides to instead select "Scientific Name," by default the boxplot legend will change to display multiple scientific names that representing the individual common name and each scientific name will be represented by a unique color/shape point on the plot. However, if the user selects the checkbox "Group by Common Name" in the "Species Legend Options" control box, then the scientific names that are represented by one common name will all display the same color/shape point on the plot.

The user has the option of removing selected species from the legend either by removing them directly from the "Select Species for Legend" drop-down box or by hovering the mouse directly over the species name in the legend. The mouse will change to a black arrow with a red 'x' next to it. Clicking the name while this arrow is displayed will remove the species from the legend and from the control box.



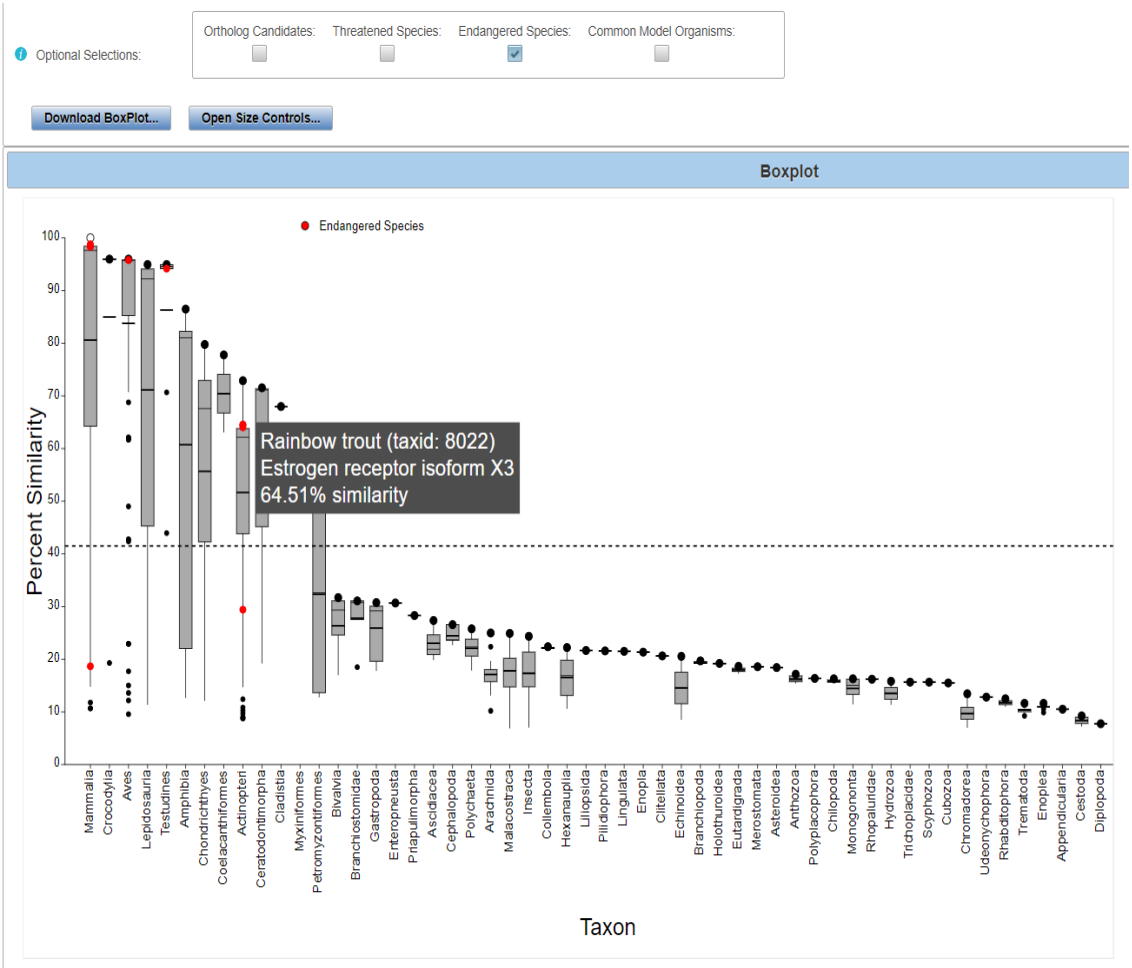


Customize the Legend to Display Species Groups of Interest

In the "Optional Selections" controls box, the user has the option of displaying "Ortholog Candidates," "Threatened Species," "Endangered Species," or "Common Model Organisms." Upon selecting one of the checkboxes, red data points corresponding to species will be displayed on the boxplot. By hovering the mouse over a single red point, a pop-up box will appear with the corresponding species name, taxonomic ID, query protein, and percent similarity.

Note: The user can select to display either species common name or scientific name in the hover over information box by selecting from the "Species Legend Options."

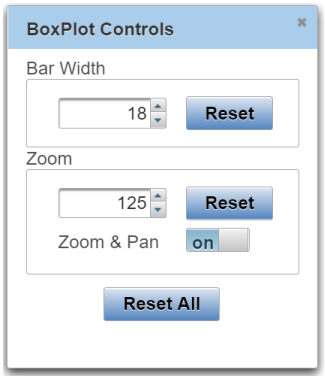
If the user selects either "Threatened Species" or "Endangered Species," clicking on an individual red dot will open a new web browser tab and link to the corresponding species page on th US Fish and Wildlife Service's Environmental Conservation Online System (USFWS, ECOS; e.g.,) (https://ecos.fws.gov/ecp0/profile/speciesProfile?sId=1506).



BoxPlot Controls Widget for Bar Width, Zoom and Pan

By clicking the "Open Size Controls" button, a "BoxPlot Controls" widget opens that allows the user to adjust the size of the bars on the boxplot by increasing or decreasing the "Bar Width" using the up and down arrows. The minimum and maximum size for bars are 6 and 60, respectively. To reset the bar width on the boxplot to default size, click the "Reset" button to the right of the "Bar Width" adjustment box in the "BoxPlot Controls" box. The user can also Zoom and Pan the boxplot by toggling the on /off button under the "Zoom" heading. The user can then zoom in or out by clicking the up or down arrows or entering a number in the text box and clicking enter. To reset the zoom on the boxplot to default size, click the "Reset" button to the right of the "Zoom" adjustment box in the "BoxPlot Controls" widget. The pan option is available when the "Zoom and Pan" option is toggled to the "on" position, which allows the user to click on the boxplot and drag the plot around the screen to reposition. To reset all BoxPlot Controls to default settings click the "Reset All" button.

Note: Upon exiting out of the BoxPlot Controls widget, the Zoom and Pan options are automatically turned off.



Download BoxPlot Widget

To download the boxplot, click "Download BoxPlot" button in the controls box. A "Download Boxplot" Widget will pop up. It will be necessary to specify which type of file (SVG, PNG, or JPG,) to downloaded by clicking on the desired radio button for "Image Type." The user may customize the resolution of the boxplot for PNG and JPG files prior to download by altering the "Width" and "Height" of the BoxPlot. To change "Width" or "Height," enter the desired number in the text boxes. Click "Download Image" button to download the file. To close the "Download Boxplot" widget, click the "x" on the top right of the widget.

Hover-over Features in the BoxPlot

By hovering over a taxonomic group name on the x-axis of the boxplot, an information box will pop-up listing the top three species in order by highest percent similarity. If only one or two species are represented in the taxonomic group, then only those species will be displayed. Hovering the mouse over any of the species in the boxplot, that is present in the legend, will generate a pop-up box with the corresponding species name, taxonomic ID, query protein, and percent similarity. The susceptiblity cut-off is displayed in a pop-up text box upon hovering over the dashed horizontal cut-off line.

Summary Table for Species in a Specific Taxonomic Group

By clicking on a box representing a taxonomic group in the boxplot a table will pop-up providing summary information for that particular group. The table header will provide summary statistics (i.e., mean and median percent similarity), including the Taxonomic Group name, number of species represented in the box, the overall susceptiblity prediciton for the selected taxonomic group. Data table includes protein and species information along with metrics for evaluated protein similarity and predicting suseptiblity. Also inlcuded in the table are columns indicating if a species belongs to a certain group of interest (e.g., Threatened Species; Endangered Species, Model Organism). Table can be downloaded by clicking on the icon for excel or csv file.

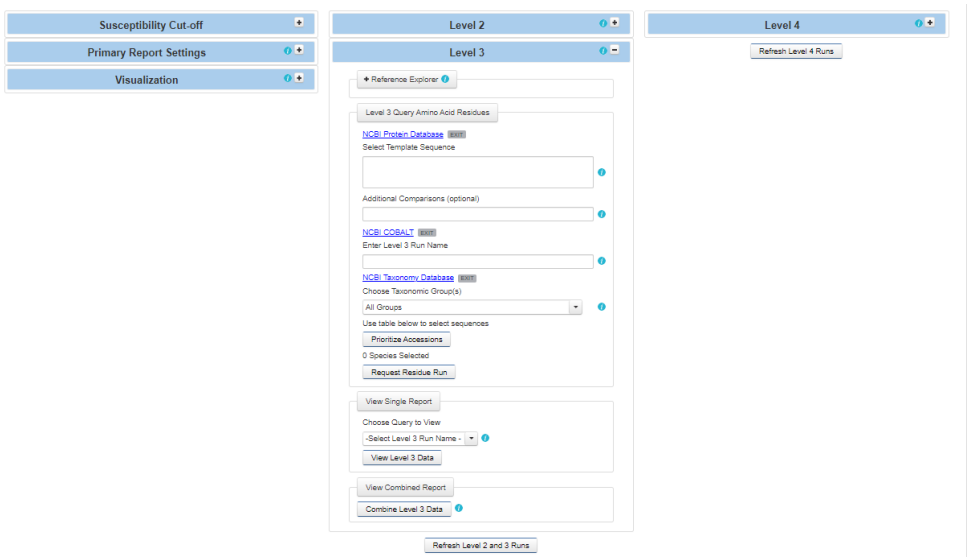
Interactive Visualization with Level 1 Data Page and Level 2 Data Page

The data visualization is programmed to update with changes made to the Level 1 Data page and Level 1 Data page, respectively. Therefore, if the user updates the Susceptibility Cut-off (See user guide section **Susceptibility Cutoff Box for Level 1** and **Susceptibility Cutoff Box for Level 2)** to the "Second Local Minimum" or "User Defined Cut-off," the previously opened data visualization boxplot tab will update the cut-off accordingly. Similarly, the user modifies the Primary Report Settings (See user guide section **Level 1: Primary Report Settings** and **Level 2: Primary Report Settings)**, the data visualization will update accordingly.

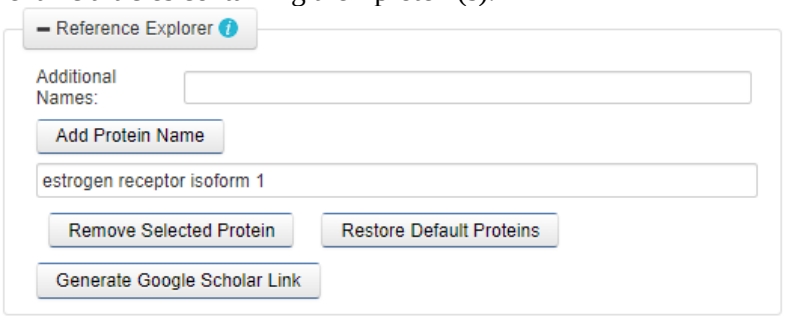
Note: If the user updates the "Primary Report Settings" for "Sorted by Taxonomic Group" the boxplot will update to display the new taxonomic group selection that is present in the "Filtered Taxonomic Group" column in the data table. The user should be aware that manipulating the "Sorted by Taxonomic Group" to a different level in the taxonomic lineage (e.g., from class to order; from class to genus) adds a larger number of taxonomic groups to the x-axis. Therefore, the plot may require greater user manipulation using the "BoxPlot Controls" to view the data.

Level 3: Individual Amino Acid Residue Alignment

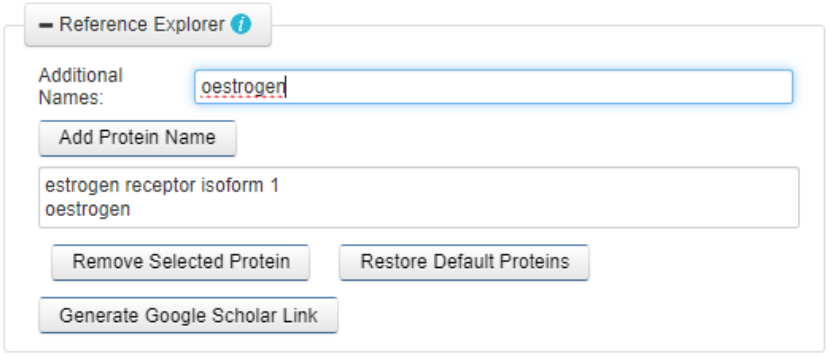
In the "View SeqAPASS Reports" tab, on the "Level 1 Query Protein Information" page, there is a "Level 3" dropdown for setting up the query for comparing individual amino acid residues to a template sequence. It is anticipated that the choice of template sequence and residues that are selected to align will be derived from the published literature in most cases. Publications evaluating homology models, protein crystal structures, pesticide field resistance, or utilizing site-directed mutagenesis are a few examples of the types of studies that may contain such information to guide a Level 3 SeqAPASS evaluation.



Relevant literature containing these data can be identified using the SeqAPASS "Reference Explorer." The user can search for literature with the protein(s) of interest with an auto-populated search term that is integrated into a predefined Boolean string and generate a Google Scholar link that will take them to scientific articles containing their protein(s).

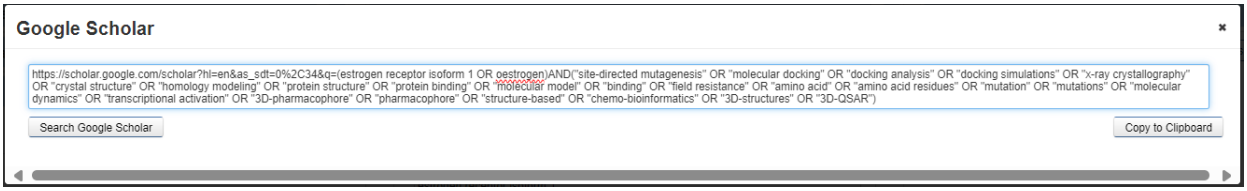


The user can modify the Boolean search string by adding text to the "Additional Names" text box and clicking the "Add Protein Name" button. By selecting a name that is currently in the text box and clicking the "Remove Selected Protein" button, the user can delete names from the text box and therefore these names will not be included in the Boolean string for the Google Scholar search.

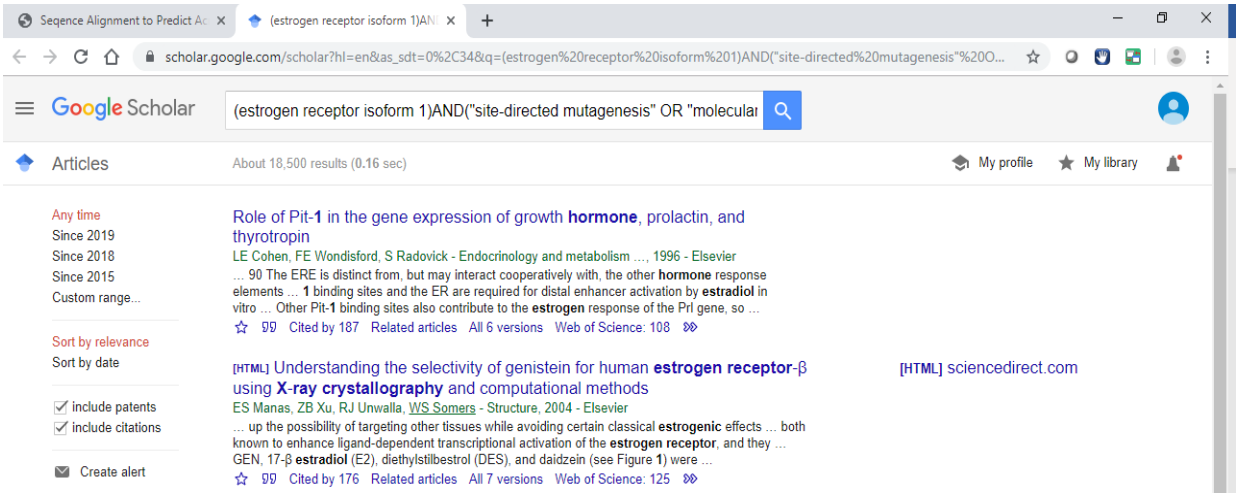


When satisfied with the protein names to be included in the Boolean search string, the user will select the "Generate Google Scholar Link" button. A pop-up will appear displaying the Boolean string to be searched in Google Scholar. The user can continue to modify the Boolean string by clicking in the text and adding additional information. The Boolean string can be copied and pasted elsewhere by the user by

clicking the "Copy to Clipboard" button. The user can also choose to use the generated Boolean string to search Google Scholar. To do so the user will select the "Search Google Scholar" button.

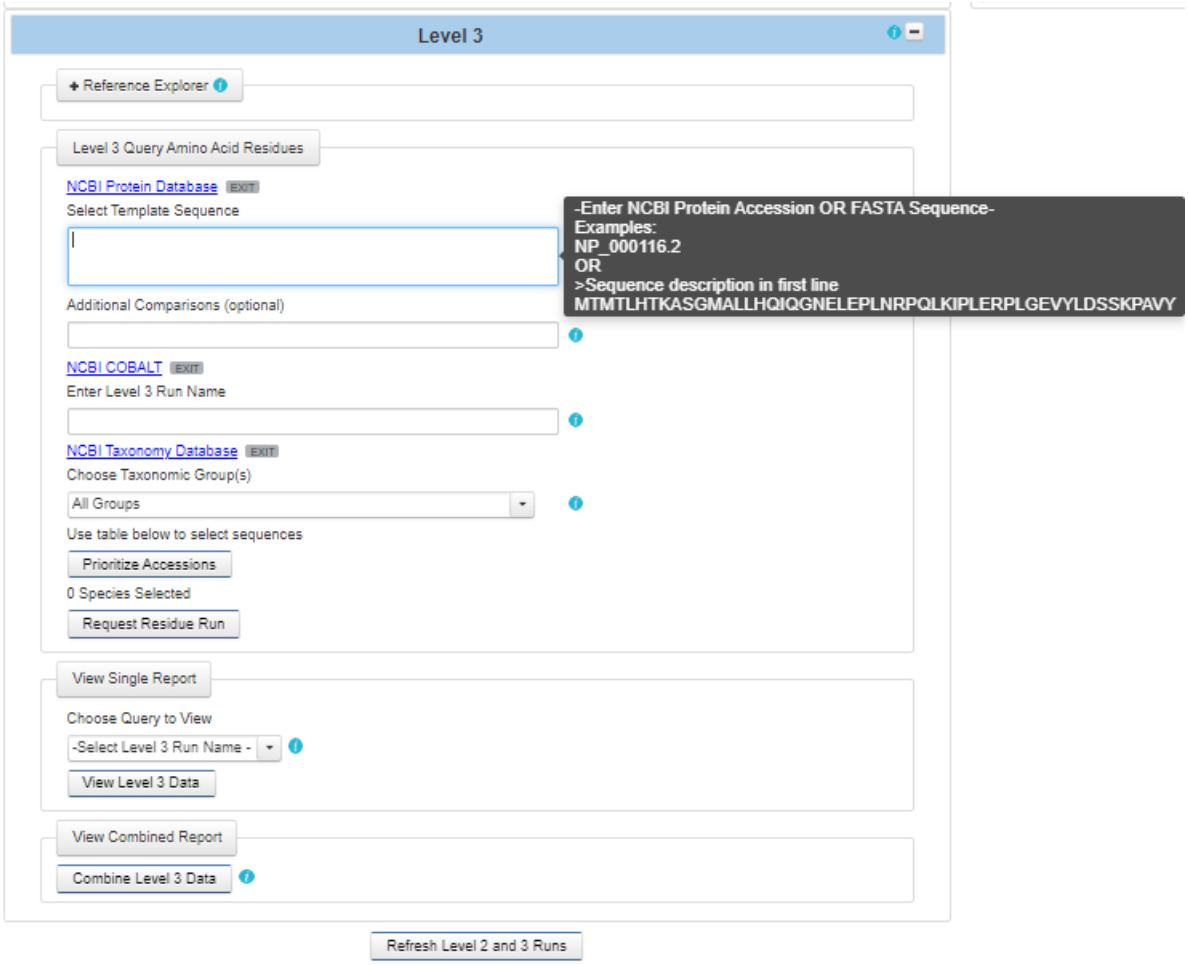


Upon selecting the "Search Google Scholar" button, a new tab will be generated in the browser for Google Scholar that contains the Boolean string in the search with publications and articles that matched the SeqAPASS generated Boolean string. The literature displayed by Google Scholar for the user should be evaluated to identify appropriate articles for determining Level 3 template sequences and critical individual amino acids for comparisons across species.



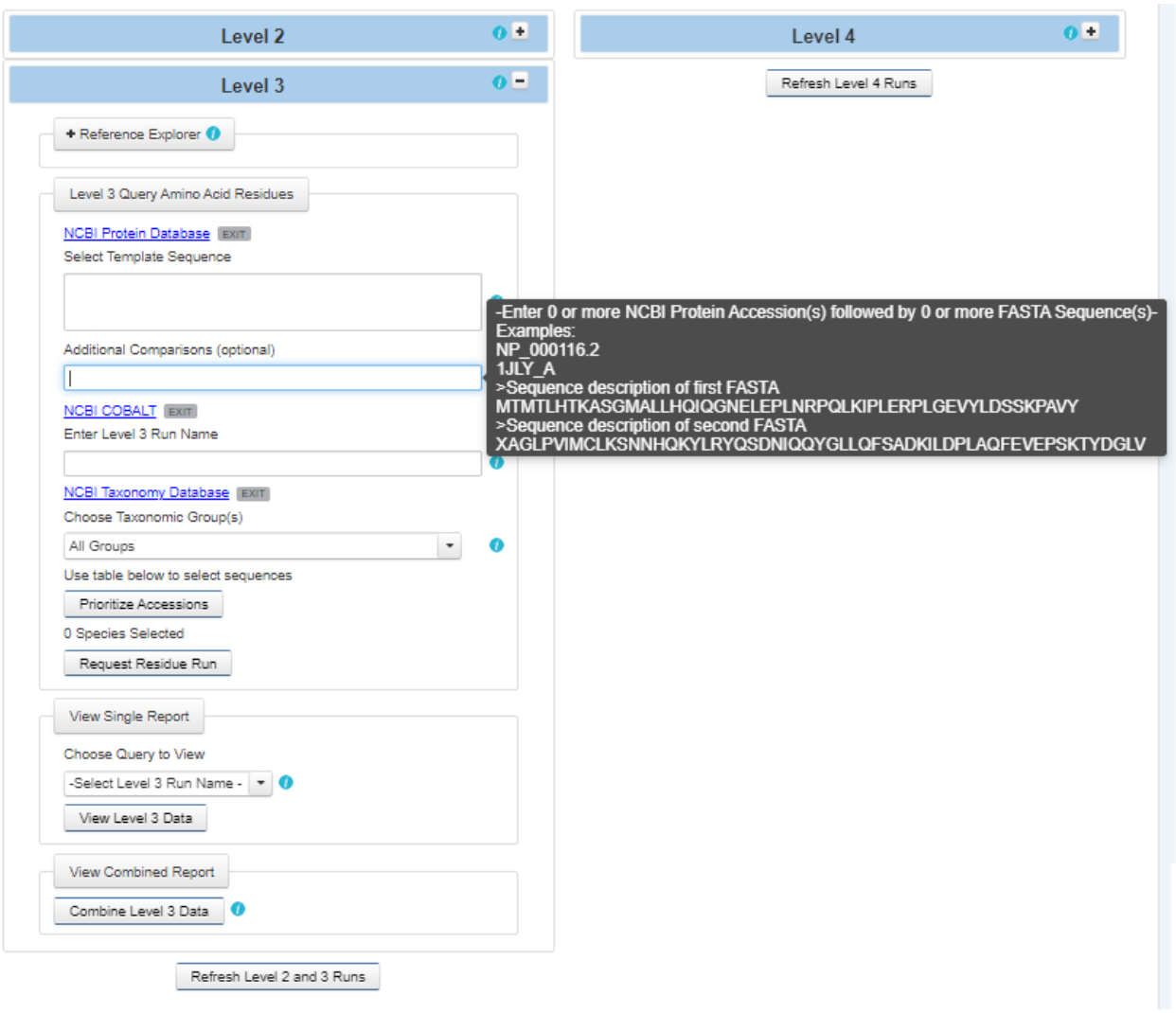
In the "Level 3" box, there is a link out to the "NCBI Protein Database" for identifying the template sequence of interest. Below this link the user will find a text box where the user can enter an NCBI Protein Accession with the version number (e.g., NP_000116.2) or a FASTA formatted sequence (e.g., < >gi|62821794|ref|NP_000116.2| estrogen receptor isoform 1 [Homo sapiens]
MTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGEVYLDSSKPAVYNYPEGAAYEFNA
AAAANA
QVYGQTGLPYGPGSEAAAFGSNGLGGFPPLNSVSPSPLMLLHPPPQLSPFLQPHGQQVPYYLENE
PSGYT
VREAGPPAFYRPNSDNRRQGGRERLASTNDKGSMAMESAKETRYCAVCNDYASGYHYGVWSC
EGCKAFFK
RSIQGHNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRD
DGEGRGEV
GSAGDMRAANLWPSPLMIKRSKKNSLALSLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMG
LLTNLA
DRELVHMINWAKRVPGFVDLTLHDQV).

Upon clicking on in the "Select Template Sequence" text box, a pop-up message will appear to provide examples for the proper format of Accessions or FASTA files to be entered. A link out to the NCBI Protein Database is available for the user and found above the template entry text box.



Additional sequences can (this is an optional field the user can choose to fill in) also be incorporated into the Level 3 alignment using the "Additional Comparisons (optional)" text box. Upon clicking on the "Additional Comparisons (optional)" text box, a pop-up message will appear to provide examples for the proper format of Accessions or FASTA files to be entered.

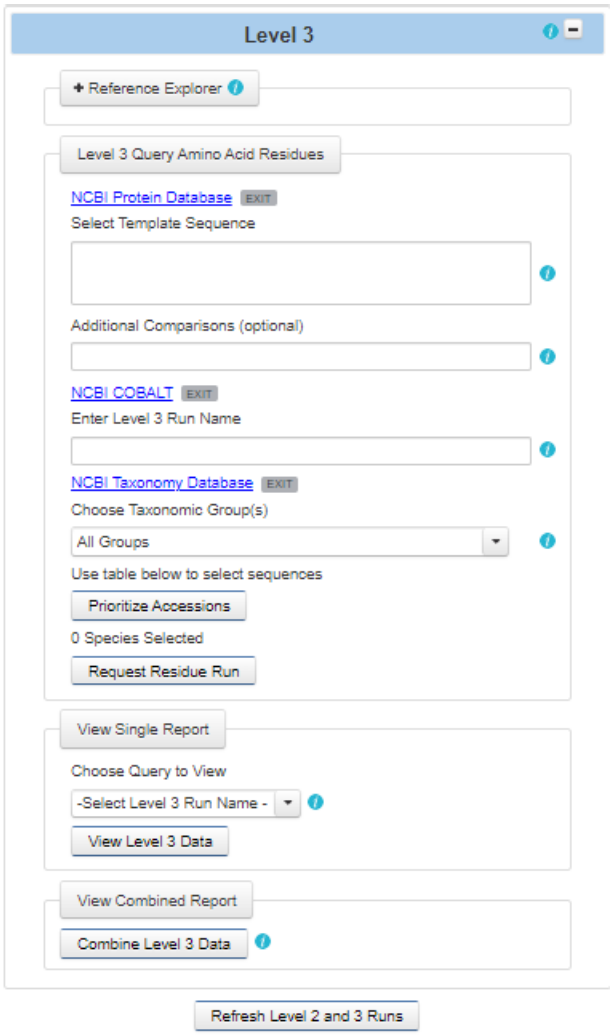
Note: In the "Additional Comparisons (optional)" text box, zero or more NCBI Protein Accession must be entered prior to FASTA sequence(s) if they are to be included in the Level 3 alignment.



Below the text box where the user can choose to add additional sequences for comparison, is a link to NCBI COBALT (Constraint-based Multiple Protein Alignment Tool). The NCBI COBALT allows the user to align multiple sequences and is the alignment tool that SeqAPASS algorithms utilize to set up the query of individual amino acid residues across species.

Note: The user does not need to use the COBALT link to run a Level 3 evaluation, however the link is available in case the user chooses to further evaluate or compare multiple potential template sequences.

Under the text "Enter Level 3 Run Name," there is a text box where the user can enter a user defined name for the run. The user may only enter letters or integers as text for the name. The user defined name will appear in the "View Level 3 Data" dropdown upon completion of the Level 3 sequence alignment.

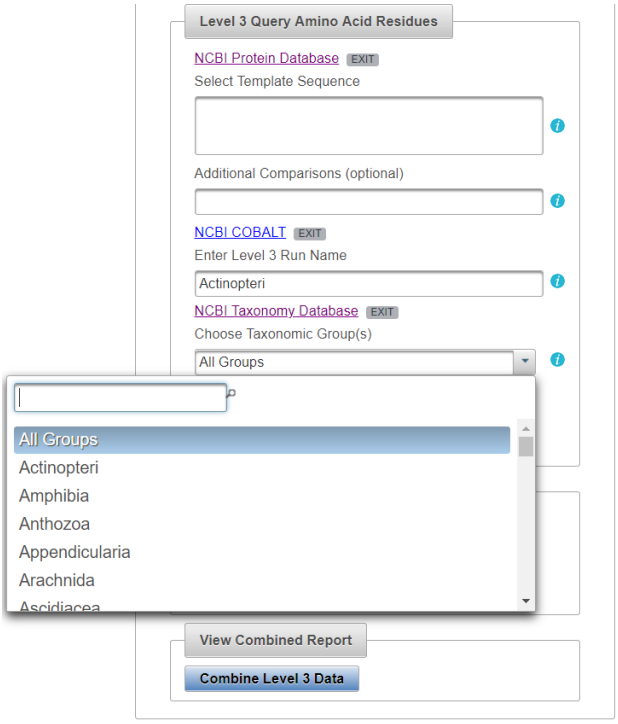


To complete the set-up for a Level 3 query the user must select which sequences to compare to the identified template sequence. Listed in the "Choose Taxonomic Group(s)" drop-down are all Taxonomic Groups that were identified as hits in the "Level 1" primary amino acid sequence alignment data. Because COBALT is used to align all sequences that are selected, it is recommended that the user selectively identify sequences from the hit table below to align. For example, selecting sequences with low similarity to the template sequence along with sequences sharing high similarity to the template sequence can skew the alignment because COBALT is trying to align all the sequences together. It is recommended that the user select sequences by first selecting a taxonomic group from the "Choose Taxonomic Group(s)" drop-down. The user can also use the NCBI taxonomy link to type in the name of the "Taxonomic Groups" found in the drop-down to look up which species fall in that group.

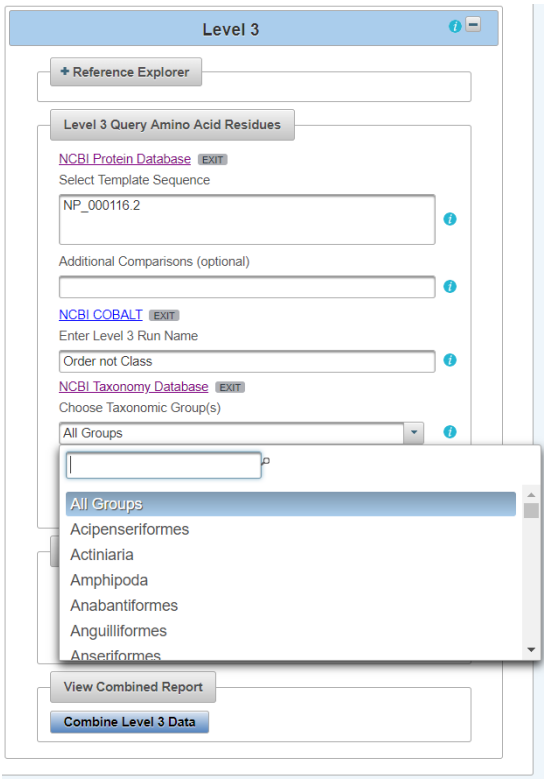
Prioritizing Accessions

The user may click the "Prioritize Accessions" button to have all accessions that meet the default standards selected, as designated by the left-hand checkbox in the Level 1 Report table. The default settings prioritize protein Accessions that begin: - NP_, O_, P_, Q_, ABC_, XP_ and deselect those that are annotated as Other Sequences, unidentified, Unclassified sequence, Bacteria, unnamed, uncharacterized, partial, hypothetical, LOW QUALITY PROTEIN, unknown, green-fluorescent protein, as these proteins typically do not provide useful results in Level 3. The user may choose to manually

update the priority selections by adding and removing accessions using the checkboxes in the first column of the Level 1 Data - Primary Report table at the bottom of the Level 1 page. After selecting "Prioritize Accessions", the number of accessions prioritized by the user will be displayed below the "Prioritize Accessions" button with the "x species selected" text. Prioritized accessions will be aligned in the Level 3 Analysis upon selecting "Request Residue Run".



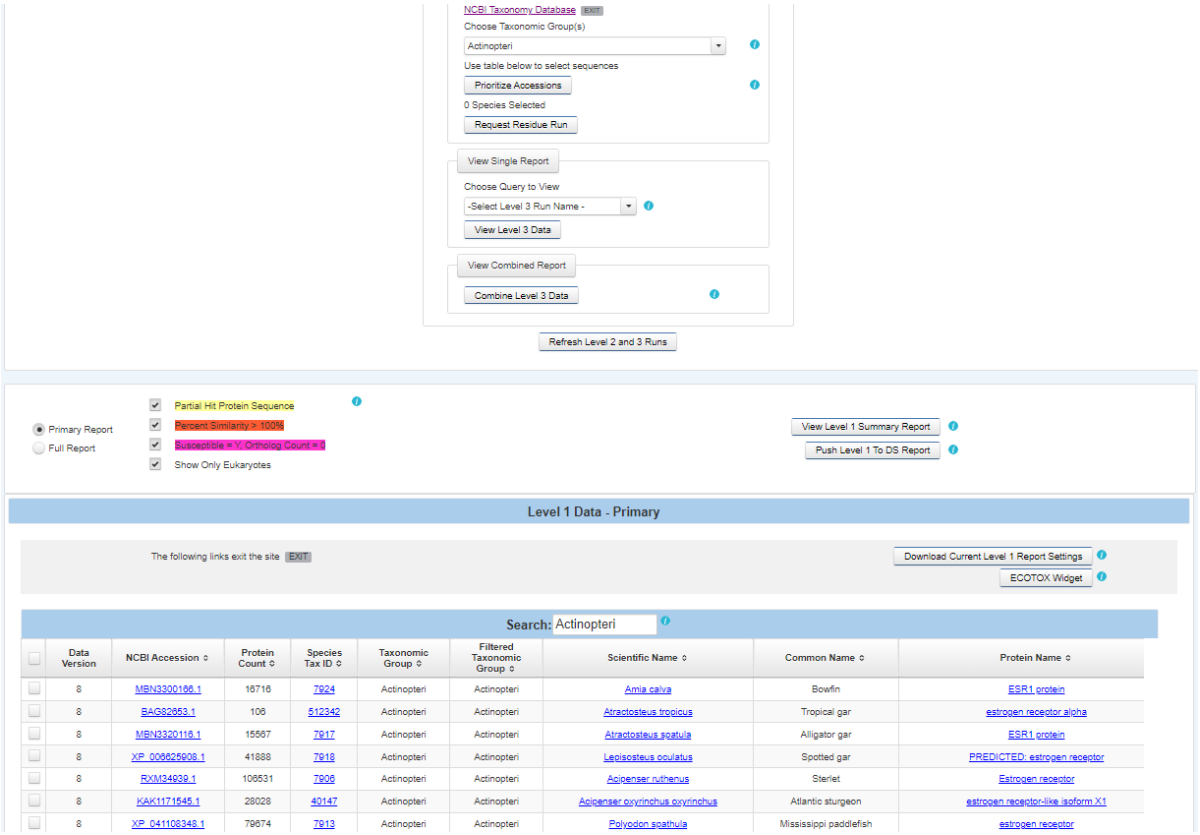
Note: The "Choose Taxonomic Group(s):" drop-down will display the level of the taxonomic hierarchy being displayed in the "Filtered Taxonomic Group" column of the "Level 1 Data" table. For example, if the user changes the default option from "class" to "order," then "order will be displayed in the dropdown.



By choosing a group from the drop-down menu, the "Level 1 Data" table below will be filtered by the selected Taxonomic Group (see column "Taxonomic Group" in "Level 1 Data" table). When a "Taxonomic Group" is selected from the drop-down, it can take up to a few seconds for the "Level 1 Data" table to filter completely, depending on the size of the table. The user can then examine each hit protein in the "Level 1 Data" table and select those that they would like to compare to the template sequence. To select sequences/species from the filtered "Level 1 Data" table, the user will select the check boxes in the first column of the table. Although it is not typically recommended, the user may also select the header check box in the first column to select all sequences/species in the filtered table.

Note: The user can also type the "Taxonomic Group" of interest in the text search box at the top of the drop-down for quick filtering.

Below is an example where the user selected the "Taxonomic Group" Actinopteri from the drop-down and then selected individual sequences/species to align with the template sequence. The number of selected species will be shown in the text above the "Request Residue Run" button.

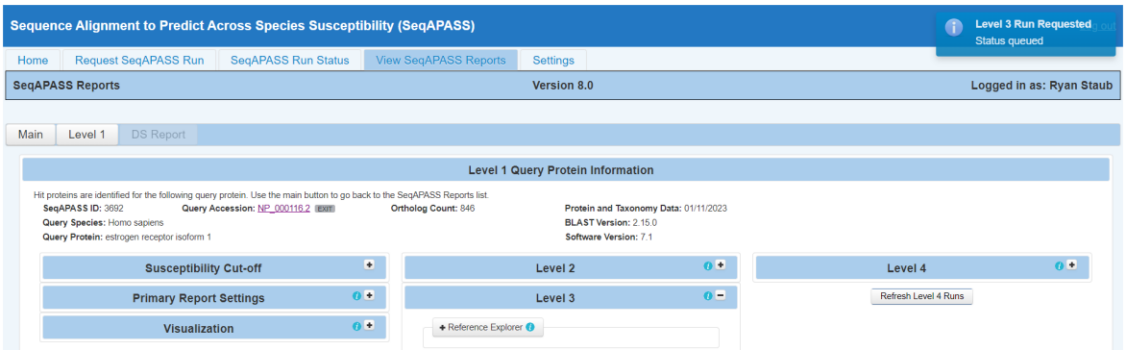


Data Version	NCBI Accession	Protein Count	Species Tax ID	Taxonomic Group	Filtered Taxonomic Group	Scientific Name	Common Name	Protein Name
8	MBN3300166.1	16716	7924	Actinopteri	Actinopteri	Amia calva	Bowfin	ESR1 protein
8	BAG82853.1	106	512342	Actinopteri	Actinopteri	Atractosteus tropicus	Tropical gar	estrogen receptor alpha
8	MBN3320116.1	15567	7917	Actinopteri	Actinopteri	Atractosteus spatula	Alligator gar	ESR1 protein
8	XP_006625908.1	41888	7918	Actinopteri	Actinopteri	Lepisosteus oculatus	Spotted gar	PREDICTED: estrogen receptor
8	RXM34939.1	106531	7906	Actinopteri	Actinopteri	Acipenser ruthenus	Sterlet	Estrogen receptor
8	KAK1171545.1	28028	40147	Actinopteri	Actinopteri	Acipenser oxyrinchus oxyrinchus	Atlantic sturgeon	estrogen receptor-like isoform X1
8	XP_041108348.1	79674	7913	Actinopteri	Actinopteri	Polyodon spathula	Mississippi paddlefish	estrogen receptor

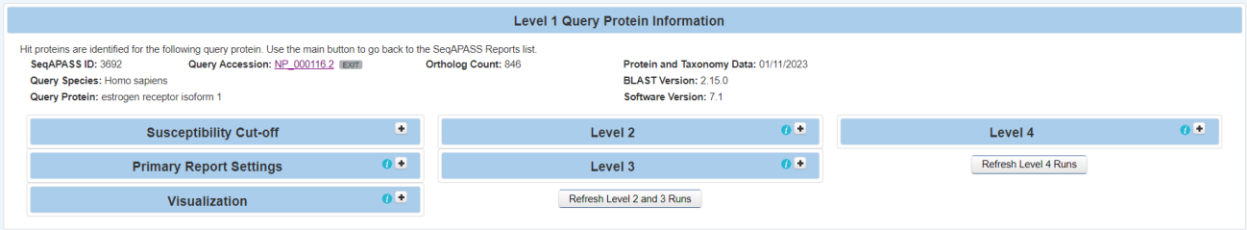
(See **Search, View, and Download Data Tables** section of user guide for more information)

The user can choose to align sequences/species from multiple taxonomic groups with the template sequence, by going back to the "Choose Taxonomic Group" drop-down and selecting another group, which filters the Level 1 table based on the group selected, and then the user can select additional species from the newly filtered table. As before, the number of selected species can be tracked in the text above the "Request Residue Run" button that reads "X species selected".

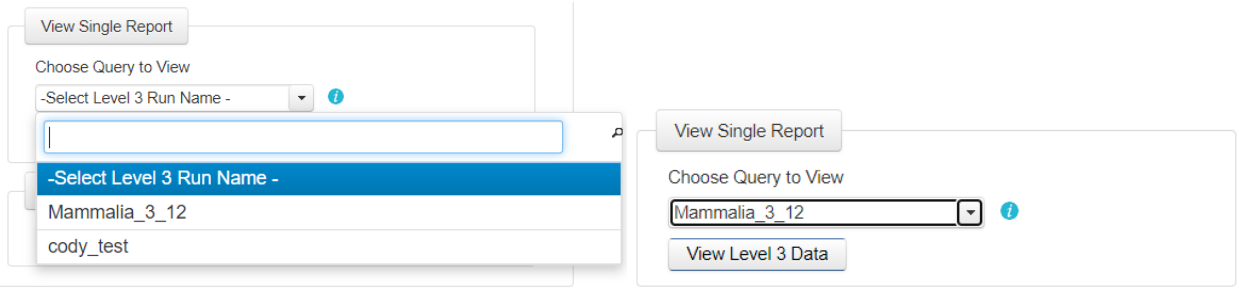
When the user has selected all sequences they want to align, then click the "Request Residue Run" button. Upon successful submission of a Level 3 query the user will see the following pop-up message. If submission is unsuccessful, a message will appear describing the reason for the unsuccessful submission.



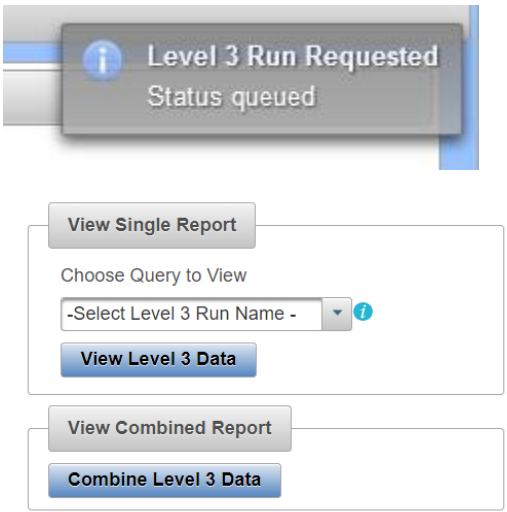
To update the "Choose Query to View" drop-down menu with the completed Level 3 alignments, the user can click on the "Refresh Level 2 and 3 runs" button.



Additionally, the user can check the status of the Level 3 run by clicking the "SeqAPASS Run Status" tab and the radio button for "Level 3 Status." Typically, Level 3 alignments complete in a few seconds. When the Level 3 query completes and the Level 1 page has been updated, the user defined Level 3 Run Name will be available in the "Choose Query to View" drop-down menu. After selecting the desired Run Name from the drop-down, click "View Level 3 Data" button to view the aligned sequences and set up the individual amino acid residue alignments with the selected sequences/species.



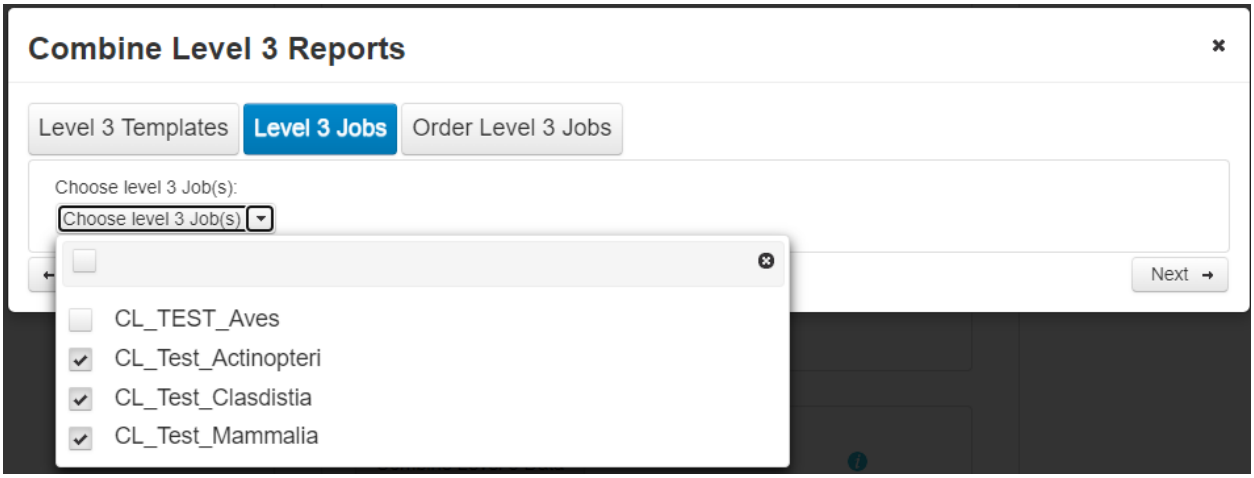
Upon a successful Level 3 query submission a pop-up message will be displayed as follows in the upper right-hand side of the screen:



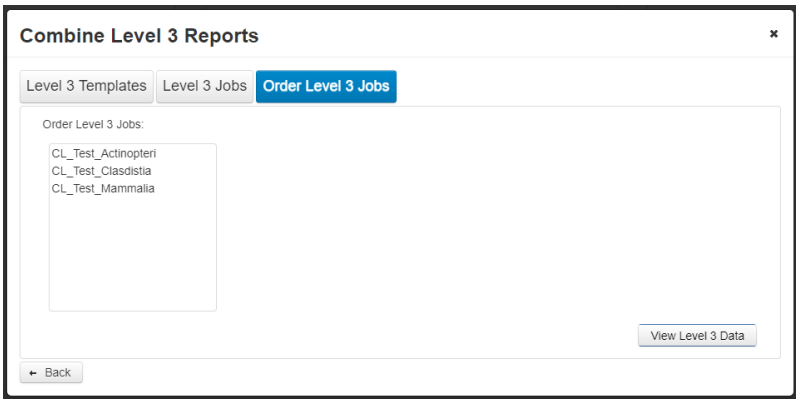
Once the Level 3 run has completed, the user can select the "Select Level 3 Run Name" drop down in the "View Single Report" box to view an individual user defined Level 3 run. If the user has completed multiple Level 3 alignments, between a template sequence and more than one taxonomic group, the user can combine Level 3 reports by selecting the "Combine Level 3 Data" button. A pop-up will appear for the "Combine Level 3 Reports". There are a series of three steps to combine Level 3 reports. First the user

will "Choose a Level 3 Template" from the dropdown that contains a list of all templates used to generate alignments in Level 3 by the user. The template sequence must be in-common to the Level 3 runs that will be combined.

After selecting the template, the user will click the "Next" button. At this point the user will select all Level 3 Jobs that are to be combined by selecting the check box in the "Level 3 Jobs" dropdown next to the user defined names. After all jobs that are to be combined are selected the user will click the "Next" button. Note that as the user moves through each step of the Combine Level 3 Reports feature, the step the user is currently on is indicated by highlighting the button in blue coloring (example "Level 3 Jobs" button is highlighted when working on selecting Jobs to combine).



The next step in the "Combine Level 3 Reports" feature is to put the jobs in order as to how they should be displayed in the output. Typically, sequences from an individual taxonomic group are aligned to a template sequence and named accordingly (e.g., Actinopteri, Amphibia, Aves, etc.). It may be useful to order the combined report similarly to how the taxonomic groups are displayed on the x-axis of the Level 1 or Level 2 data visualization. Therefore, the user can select the user defined name from the "Order Level 3 Jobs*:"* text box and drag and drop the name to the desired order from top to bottom. To move on to select individual amino acids for sequence comparisons the user will select the "View Level 3 Data" button.



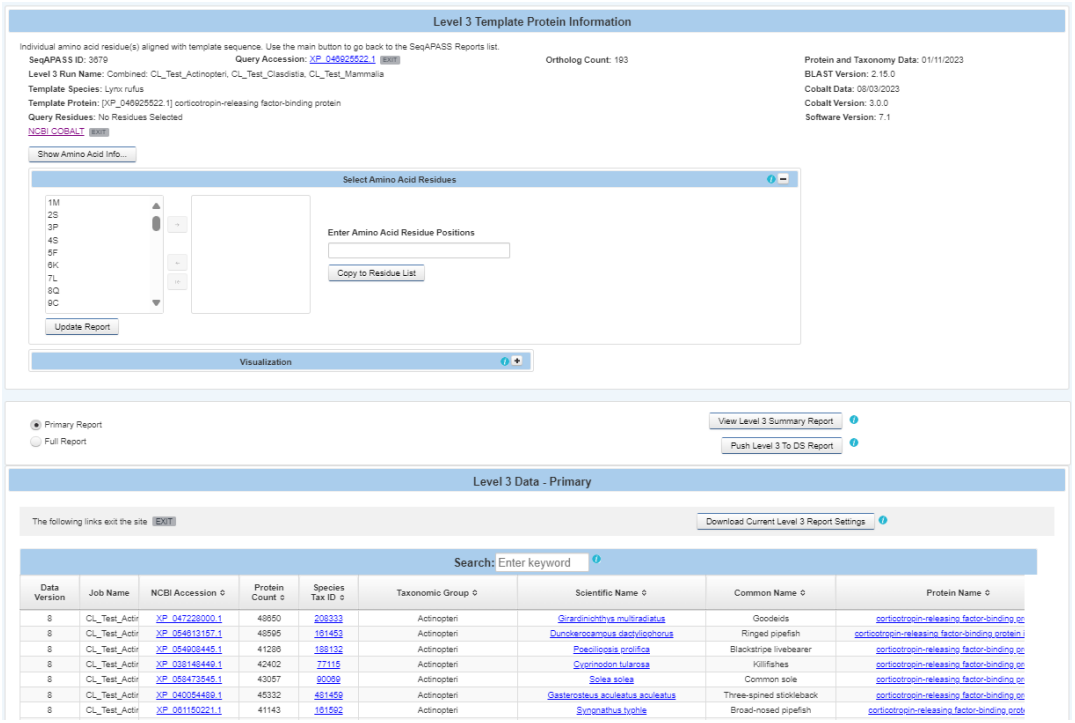
The order selected will translate to the top to bottom order displayed in the data table, with the template sequence only displayed once in the first row and all selected jobs below.

Search: Enter keyword

Data Version	Job Name	NCBI Accession	Protein Count	Species Tax ID	Taxonomic Group	Scientific Name	Common Name	Protein Name
8	CL_Test_Actir	XP_047228000.1	48650	208333	Actinopteri	Girardinichthys multiradiatus	Goodeids	corticotropin-releasing factor-binding prot
8	CL_Test_Actir	XP_054613157.1	48595	161453	Actinopteri	Dunckerocampus dactyliophorus	Ringed pipefish	corticotropin-releasing factor-binding protein is
8	CL_Test_Actir	XP_054908445.1	41286	188132	Actinopteri	Poeciliopsis prolifica	Blackstripe livebearer	corticotropin-releasing factor-binding prot
8	CL_Test_Actir	XP_038148449.1	42402	77115	Actinopteri	Cyprinodon tularosa	Killifishes	corticotropin-releasing factor-binding prot
8	CL_Test_Actir	XP_058473545.1	43057	90069	Actinopteri	Solea solea	Common sole	corticotropin-releasing factor-binding prot
8	CL_Test_Actir	XP_040054489.1	45332	481459	Actinopteri	Gasterosteus aculeatus aculeatus	Three-spined stickleback	corticotropin-releasing factor-binding prot
8	CL_Test_Actir	XP_061150221.1	41143	161592	Actinopteri	Syngnathus typhle	Broad-nosed pipefish	corticotropin-releasing factor-binding protei
8	CL_Test_Actir	XP_049595129.1	47901	161590	Actinopteri	Syngnathus scovelli	Gulf pipefish	corticotropin-releasing factor-binding prot
8	CL_Test_Actir	XP_043868495.1	91952	28829	Actinopteri	Solea senegalensis	Senegalese sole	corticotropin-releasing factor-binding protein is
8	CL_Test_Actir	XP_051907637.1	42976	109293	Actinopteri	Hippocampus zosterae	Dwarf seahorse	corticotropin-releasing factor-binding protei
8	CL_Test_Clas	XP_039614339.1	64397	55291	Cladistia	Polypterus senegalus	Gray bichir	corticotropin-releasing factor-binding prot
8	CL_Test_Mam	XP_045352099.1	67612	46844	Mammalia	Leopardus geoffroyi	Geoffroy's cat	corticotropin-releasing factor-binding protein is
8	CL_Test_Mam	XP_042815309.1	56149	9694	Mammalia	Panthera tigris	Tiger	corticotropin-releasing factor-binding prot
8	CL_Test_Mam	XP_042762553.1	53687	9689	Mammalia	Panthera leo	Lion	corticotropin-releasing factor-binding prot
8	CL_Test_Mam	XP_060491502.1	55633	9690	Mammalia	Panthera onca	Jaguar	corticotropin-releasing factor-binding protein is
8	CL_Test_Mam	XP_053763236.1	57098	9691	Mammalia	Panthera pardus	Leopard	corticotropin-releasing factor-binding protein is
8	CL_Test_Mam	XP_049505635.1	44053	29064	Mammalia	Panthera uncia	Snow leopard	corticotropin-releasing factor-binding protein is

View Level 3 Individual Amino Acid Query and Data Page

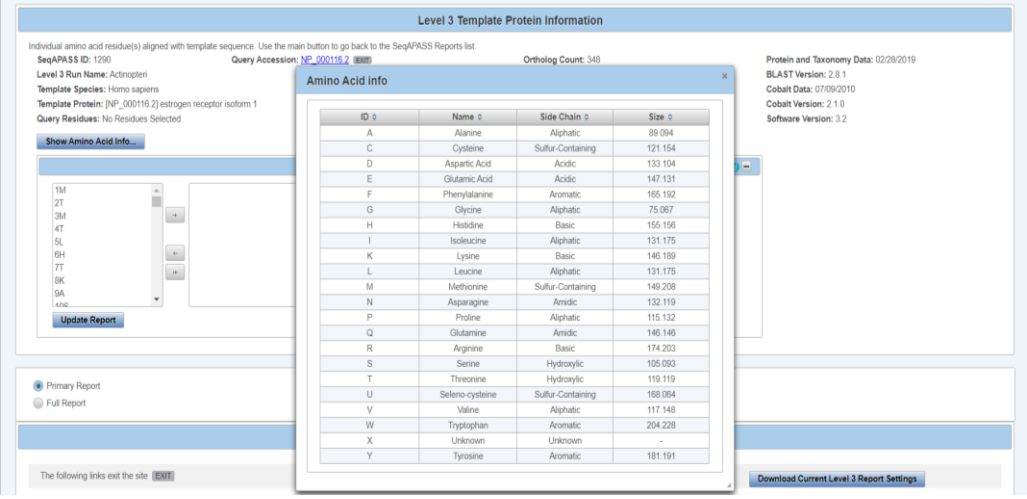
Clicking the "View Level 3 Data" button, the Level 3 data page opens. The "Level 3 Template Protein Information" box contains the SeqAPASS Run ID, Query Accession (with link out to NCBI), Ortholog Count (# of hits identified as ortholog candidates to the query species protein sequence), NCBI Data (displays the date that NCBI databases and executables were downloaded and incorporated into SeqAPASS), Level 3 Run Name (defined by user), Template Species (Entered by user in Level 3 query), Template Protein, and Query Residues (this field is populated with residues upon selection and successful table update).



The user can view the "Level 3" data page, which includes the Data Version, NCBI Accession, Protein Count, Taxonomic information, Protein Name, and date/time the Level 3 run completed. The data table remains in order of percent similarity, with those sequences having the highest percent similarity to the template sequence, on the top, to those with the lowest percent similarity on the bottom. (See **Search,**

View, and Download Data Tables section of user guide for more information.

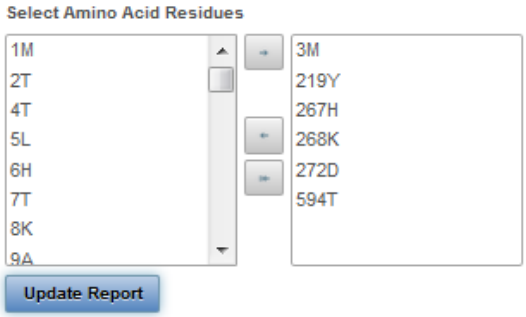
For additional information on Amino Acid Residues, including definition of the acronym, the amino acid residue name, the classification for the amino acid side chain and the size of the amino acid residue based on molecular weight, the user can click the "Show Amino Acid Info…" button. A pop-up table, "Amino Acid info," will be displayed providing this information.



ID	Name	Side Chain	Size
A	Alanine	Aliphatic	89.094
C	Cysteine	Sulfur-Containing	121.154
D	Aspartic Acid	Acidic	133.104
E	Glutamic Acid	Acidic	147.131
F	Phenylalanine	Aromatic	165.192
G	Glycine	Aliphatic	75.067
H	Histidine	Basic	155.156
I	Isoleucine	Aliphatic	131.175
K	Lysine	Basic	146.189
L	Leucine	Aliphatic	131.175
M	Methionine	Sulfur-Containing	149.208
N	Asparagine	Amidic	132.119
P	Proline	Aliphatic	115.132
Q	Glutamine	Amidic	146.146
R	Arginine	Basic	174.203
S	Serine	Hydroxylic	105.093
T	Threonine	Hydroxylic	119.119
U	Seleno-cysteine	Sulfur-Containing	168.064
V	Valine	Aliphatic	117.148
W	Tryptophan	Aromatic	204.228
X	Unknown	Unknown	-
Y	Tyrosine	Aromatic	181.191

To obtain individual amino acid residue alignment data in the Level 3 data table, the user must use the shuttle in the "Level 3 Template Protein Information" box to select positions and amino acid residues from the chosen template sequence to align with the sequences/species that were selected by taxonomic group. Single letter abbreviations are used for the amino acid sequences.

G: Glycine	A: Alanine	S: Serine	T: Threonine	C: Cysteine	V: Valine
L: Leucine	I: Isoleucine	M: Methionine	P: Proline	F Phenylalanine	U: Seleno-cysteine
Y: Tyrosine	W: Tryptophan	D: Aspartic Acid		E: Glutamic Acid	
N: Asparagine	Q: Glutamine	H: Histidine	K: Lysine	R: Arginine	

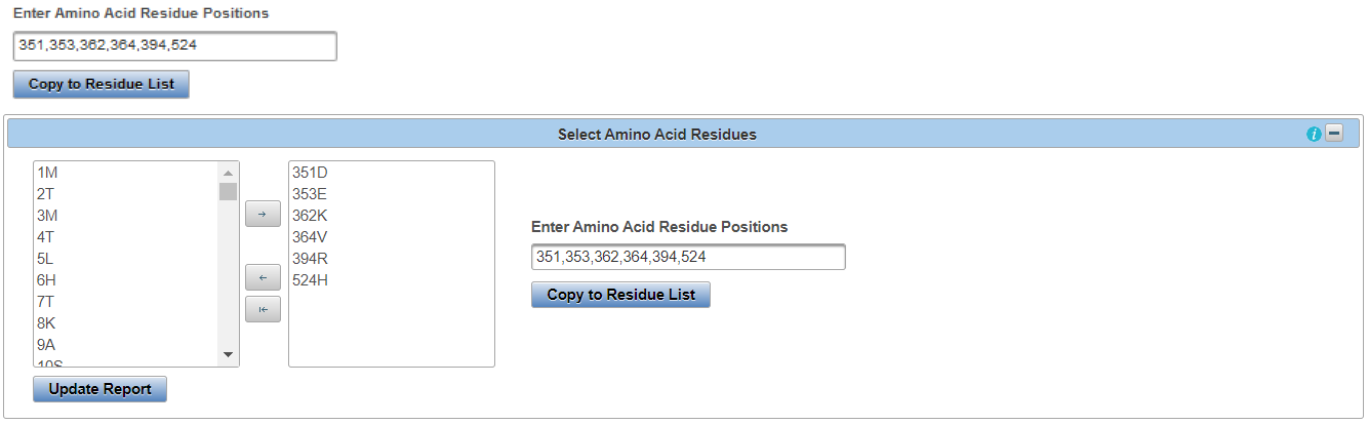


The user can select one residue at a time by clicking and highlighting the residue of interest and then clicking the top right arrow shuttle button to move the residue to the right-hand box for inclusion in the alignment. Each time a residue is added to the right-hand box, the left-hand box resets itself to the 1st residue. Or the user can select multiple residues at the same time by holding the Ctrl button, clicking on residues, and then clicking the top right arrow shuttle button to move the residues to the right-hand box. The user can choose to remove selected residues by using the left arrow button to clear one at a time or the double left arrow button to remove all selected residues at once. When residues of interest (likely

defined from the literature as described above) have been selected, click the "Update Report" button, which then updates the "Level 3 Data" table with the individual residue alignment data.

Alternatively, the user can enter the amino acid positions in the "Enter Amino Acid Residue Positions" text box (e.g., 351,353,362) and click the "Copy to Residue List" button.

Upon clicking "Copy to Residue List" the "Select Amino Acid Residues" shuttle box is populated with the position and residues typed. The user can then click the update Report button to produce Level 3 results in the table below.

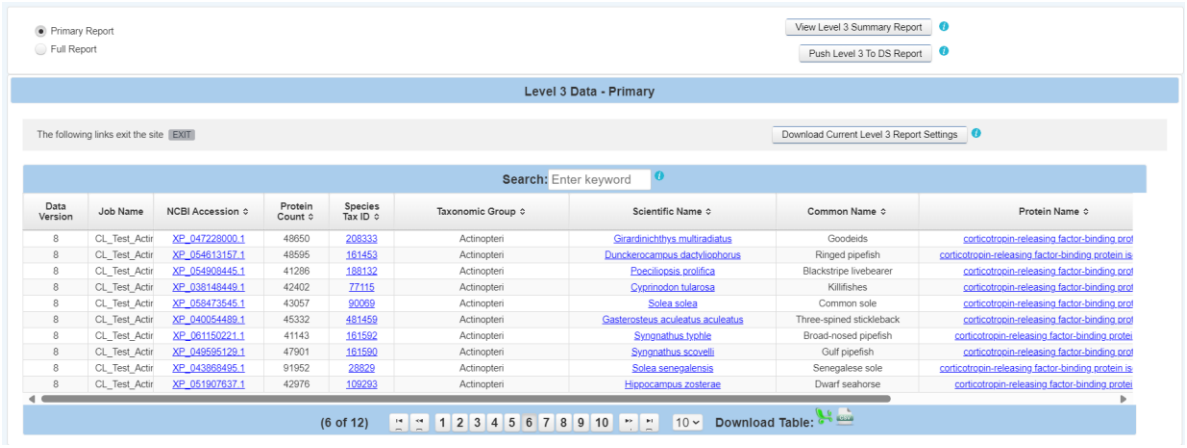


The individual amino acid residue alignment data will then be updated on the right most columns of the Level 3 Data table. The user ***can submit a maximum of 50 individual amino acid residues*** from the template sequence to compare to the other selected sequences. The individual amino acid residues will be listed in numerical order starting with the 1st position in the template sequence to the last position in the template sequence.

Level 3 Data – Primary Report

The default report is the "Primary Report" and can be recognized as such because the radio button for "Primary Report" above the "Level 3 Data" table is selected.

The "Primary Report" columns for the alignment will be titled "Similar Susceptibility as Template" ("Y" or "N" for yes or no, respectively), followed by Position 1, Amino Acid 1, Total Match 1, Position 2 Amino Acid 2, Total Match 2, Position 3, Amino Acid 3, Total Match 3…. The template sequence will always be in the top row of the "Level 3 Data" table followed by the previously selected sequences. Further, the residues selected in the shuttle will also be displayed in the top row corresponding to the template sequence. Each Position and Amino Acid in the following rows are those corresponding to the Protein Accession identified in that row and aligning with the template sequence. The Total Match X describes whether the amino acid residue matches the template based on side-chain classification and molecular weight, "Y," for yes, or "N," for not a match to the template. The user can evaluate this data to understand how well conserved an amino acid residue is across species or in a species of interest to add an additional line of evidence to support (or question) susceptibility predictions. The user can also download the current report settings by selecting the "Download Current Level 3 Report Settings." This csv allows the user to track which settings were used or changed by the user when downloading a data table.



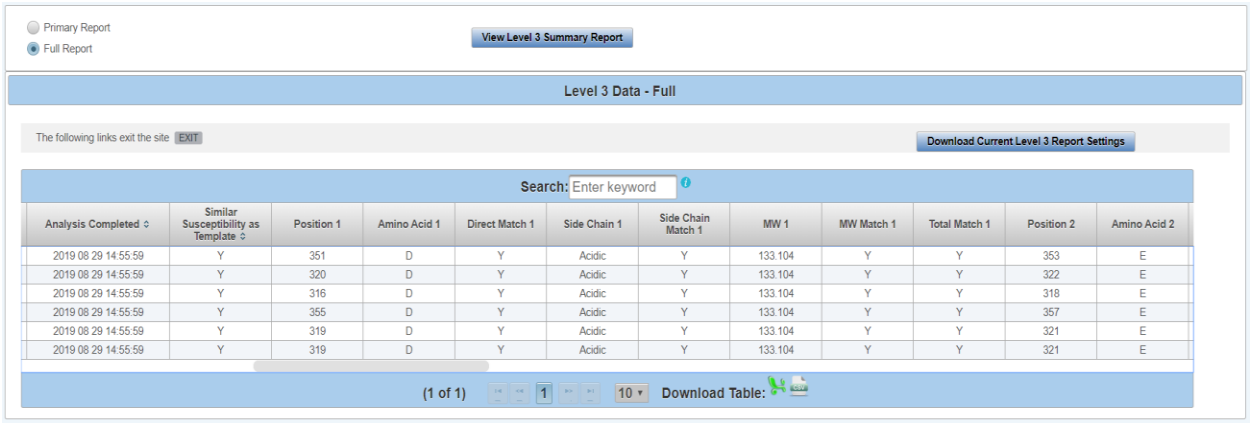
When downloading the current "Level 3 Report Settings", the following information will be present in the csv. If the user decides to change the default settings, the csv can be utilized for quick information if the SeqAPASS page is no longer open.

	A	B
1	Level 3 Report Settings	
2		
3		
4	Analysis TimeStamp	2019 05 16 11:04:08
5	SeqAPASS version	3.2
6	Level 3 Run Name	Actinopteri
7	Template Species	Homo sapiens
8	Template Protein	[NP_000116.2] estrogen receptor isoform 1
9	Query Residues	1M, 2T, 3M, 4T, 5L, 6H, 7T, 8K, 9A, 10S
10	Query Accession	NP_000116.2

Level 3 Data – Full Report

The user may choose to view the Full Report for Level 3 data by selecting the radio button above the "Level 3 Data" table for "Full Report." The table below will automatically update to display all of the alignment details.

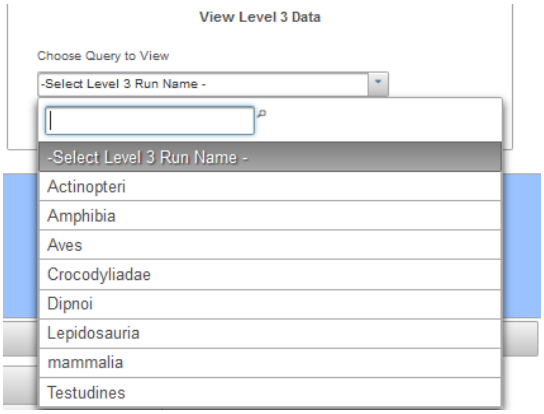
The "Full Report" columns for the alignment will be titled "Similar Susceptibility as Template" ("Y" or "N" for yes or no respectively), followed by Position 1, Amino Acid 1, Direct Match 1, Side Chain 1, MW1, MW Match 1Total Match 1, Total Match 1, Position 2, Amino Acid 2, Direct Match 2, Side Chain 2, MW2, MW Match Total Match 2, Total Match 2…… The template sequence will always be in the top row of the "Level 3 Data" table followed by the previously selected sequences. Further, the residues selected in the shuttle will also be displayed in the top row corresponding to the template sequence. Each Position and Amino Acid in the following rows are those corresponding to the Protein Accession identified in that row align with the template sequence. The Total Match X describes whether the amino acid residue matches the template based on side-chain classification and molecular weight, "Y," for yes, or "N," for not a match to the template. The user can evaluate this data to understand how well conserved an amino acid residue is across species or in a species of interest to add an additional line of evidence to support (or question) susceptibility predictions.



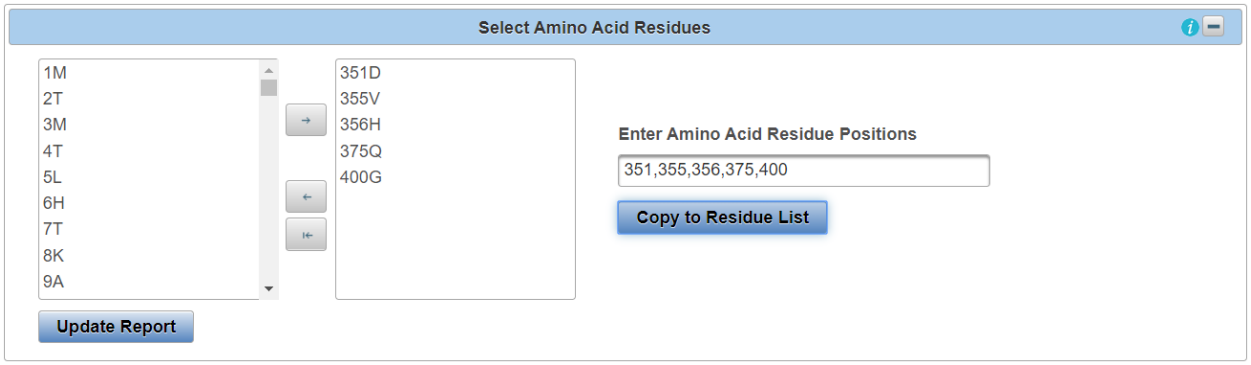
The "Direct Match X" column describes whether the hit amino acid is an exact match to the template amino acid, providing a "Y" or "N" for yes or no, respectively. The "Side Chain X" column indicates the side chain classification for the amino acid residue (click on "Show Amino Acid Info…for more information on classifications). The "Side Chain Match X" column indicates whether the hit side chain has the same classification as the template amino acid, providing a "Y" or "N" for yes or no, respectively. The "MW X" column indicates the molecular weight (g/mol) of the amino acid residue and the "MW Match X" column indicates whether the hit molecular weight has a difference in molecular weight greater than or equal to 30 g/mol compared to the template amino acid, providing a "Y" or "N" for yes or no, respectively. For the "Total Match X" to be "Y," both "Side Chain Match X" and "MW Match X" should be either "Y" and Y" or one "Y" and one "N," respectively. Only if both "Side Chain Match X" and "MW Match X" are "N" and "N," then the "Total Match X" is "N" for no. Ultimately, the Total Match 1, 2, 3, 4…. are used to inform the "Similar Susceptibility as Template" column. If there is one or more "N" for Total Match comparing any amino acid residue to the template across a row for a given species, then the "Similar Susceptibility as Template" is "N" for no, indicating that the hit species is predicted NOT to have the same susceptibly prediction as the template sequence. However, if all "Total Match X" are "Y" for yes, then the "Similar Susceptibility as Template" is "Y" indicating that the hit species is predicted to have the same susceptibly prediction as the template sequence.

Multiple Level 3 Runs Requiring the Same Amino Acid Residue Comparisons

Typically, Level 3 individual amino acid residue alignments are submitted repetitively, comparing species from one taxonomic group at a time to the template amino acid residue(s).



Therefore, to increase efficiency in submitting the same alignments in Level 3 over and over again, the user can take advantage of the "Copy to Residue List" button. For the first alignment of amino acid residues, the user would select the amino acid residues to align and click the "Update Report" button.



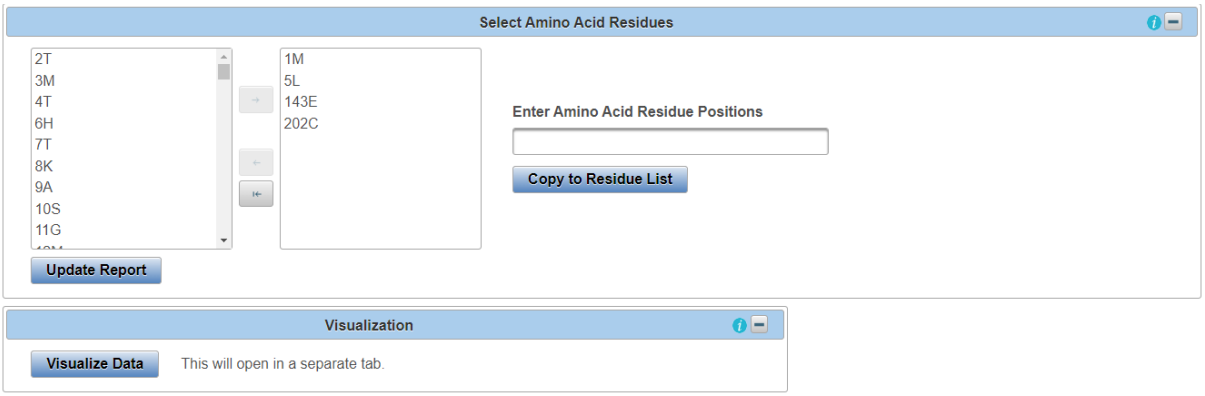
By clicking "Update Report" the residues that were selected will be copied into the "Enter Amino Acid Residue Positions" text box. When the user selects a new "Level 3 Run Name" (from the same Level 1 query accession) to view by using the "View Level 3 Data" dropdown and clicking the "View Level 3 Data" button on the "Level 1 Query Protein Information" page, the "Enter Amino Acid Residue Positions" text box will be populated with the amino acid residues selected from the previous run.

The user can keep, add, or delete, residue positions in this box and click "Copy to Residue List" button. The amino acid residues will then be moved to the "Select Amino Acid Residues Shuttle" and the user can then click "Update Report" to view the data in the table below.

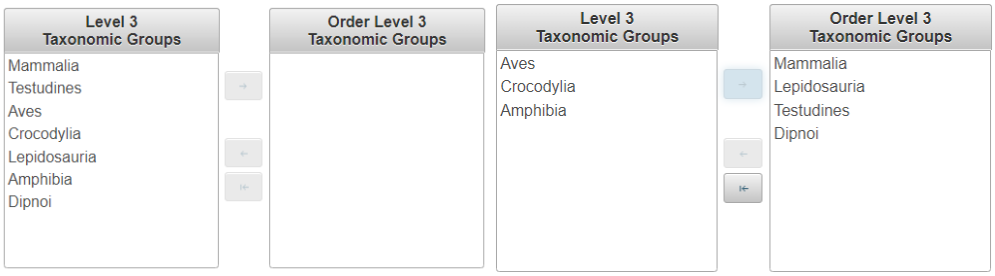
Heat Map

The Heat Map is a feature that allows the user to have a visual representation of the chosen amino acid(s) for a single Level 3 run. The Heat Map utilizes color to denote which amino acids are a total match, partial match and not a match to the template sequence. The Heat Map is accessed within the "Level 3" page under the "Visualization" drop down and will open up in a separate tab. The Heat Map has many similar features to the Level 1 and 2 boxplots with some added customizable features. There are many settings that can be changed within the Heat Map and if necessary, there are informational buttons that can be opened to get added information regarding the different options.

To get to the Heat Map, open a completed Level 3 run and click the "Visualization" drop down then select the "Visualize Data" button. This will bring you to the Heat Map where there is information regarding the features of the map. Then select the "Heat Map" icon to access the Heat Map itself.



The default order of the taxonomic groups is based on how the species are selected during the Level 3 set up process. There is the option to include all taxonomic groups or a user chosen few. To move the taxonomic groups over to place them in order you must either click or *CTRL* click and select the arrow pointed to the right. Once the taxonomic groups are moved over, the user can order the groups by dragging them up or down.



Report Options

There are multiple options within the Heat Map that can be changed based on what information the user desires to have present. The Heat Map itself can be changed between the "Simple" report which shows the amino acid and its respective position or the "Full" report which gives added information about each amino acid. The user can also change between the common name and scientific name displayed on the Heat Map.

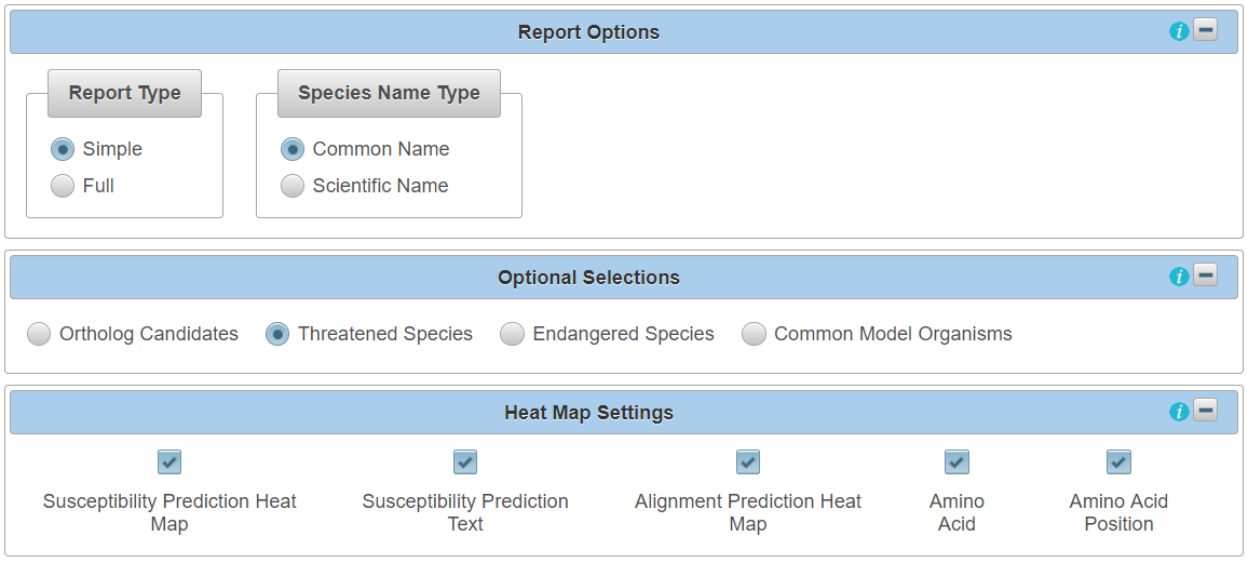
Optional Selections

The "Optional Selections" for the Heat Map will highlight the name for each respective species based on what is selected; Ortholog Candidates, Threatened Species, Endangered Species, Common Model Organism. Only one optional selection can be highlighted at a time.

Common Name	Amino Acid 1	Amino Acid 2	Amino Acid 3	Amino Acid 4
Human	32K	46S	55P	64A
Diamondback terrapin	32K	46S	55P	64T
Western painted turtle	32K	46S	55P	64T
Chinese soft-shelled turtle	32K	46S	55P	64T
Terrapins	32K	46S	55P	64T
Goodes thornscrub tortoise	32K	46S	55P	64T
Pacific ridley	32K	46S	55P	64T
Painted turtle	32K	46S	55P	64T
Green sea turtle	32K	46S	55P	64T
Three-toed box turtle	61K	75S	84P	93T

Heat Map Settings

Changing the "Heat Map Settings" will give the user the option to display specific information in the Heat Map. The user can select or deselect a variety of the settings to have a customized Heat Map.



Common Name	Similar Susceptibility	Amino Acid 1	Amino Acid 2	Amino Acid 3	Amino Acid 4
Human	Y	133E	135E	137S	584I
Western painted turtle	Y	127E	129D	131S	577V
Lappet-faced vulture	N	125E	127E	129G	575I
Nile crocodile	Y	127E	129D	131S	575I
Split-tongued squamates	Y	127E	129E	131N	576V
Japanese giant salamander	N	126E	128E	130G	573L
West African lungfish	Y	131E	133E	135S	580G

Total Match
Partial Match
Not a Match

Above is an example of a simple report which shows the amino acid and its respective position. Each amino acid is compared to the template species and can receive a dark blue color (Total match), a light blue color (Partial match), or a yellow color (Not a match). To access more information regarding each amino acid, the user can scroll over the amino acid box to bring up a box with added data.

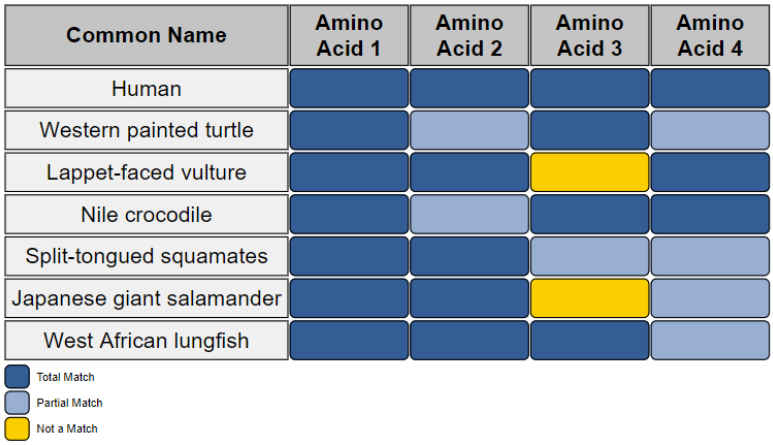
How amino acids are compared to the template: Comparing Side Chain Classification (acidic, basic, aromatic, etc.) and Molecular weight as surrogate for size (> 30g/mol different in size). Both the same (total match), One the same (partial match), Both differ (not a match).

Below is an example of a full report which also shows the amino acid and its respective position but also shows the amino acid's side chain, molecular weight and if it is a Total match (dark blue) or Not a match (yellow) to the template species.

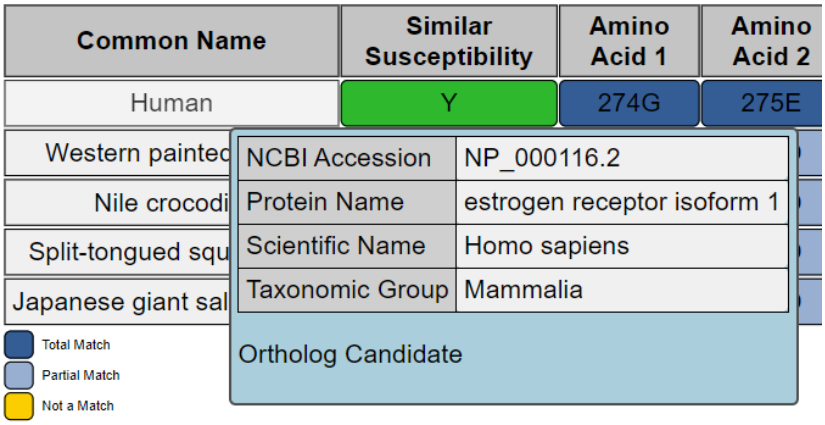
Common Name	Similar Susceptibility	Amino Acid 1	Side Chain 1	MW 1	Total Match 1	Amino Acid 2	Side Chain 2	MW 2	Total Match 2	Amino Acid 3	Side Chain 3	MW 3	Total Match 3
Human	Y	274G	Aliphatic	75.067	Y	275E	Acidic	147.131	Y	276G	Aliphatic	75.067	Y
Western painted turtle	N	268Q	Amidic	146.146	N	269D	Acidic	133.104	Y	270A	Aliphatic	89.094	Y
Nile crocodile	N	268Q	Amidic	146.146	N	269D	Acidic	133.104	Y	270A	Aliphatic	89.094	Y
Split-tongued squamates	N	268Q	Amidic	146.146	N	269D	Acidic	133.104	Y	270S	Hydroxylic	105.093	N
Japanese giant salamander	N	267P	Aliphatic	115.132	Y	268D	Acidic	133.104	Y	269Q	Amidic	146.146	N

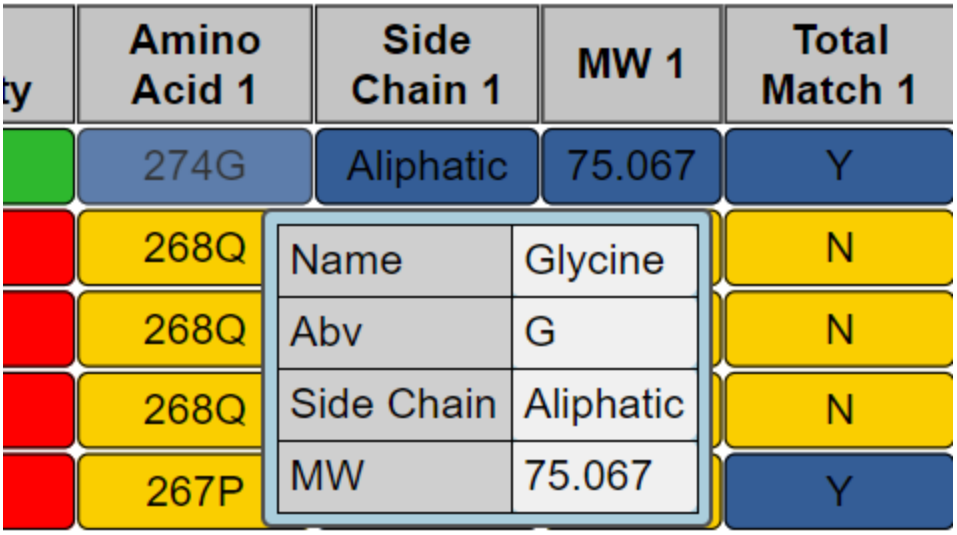
Match
Not a Match

The example below shows only the "Alignment Prediction" (Amino acid match against template) for each amino acid in chronological order.



There is added information for each species (NCBI Accession, Protein Name, Scientific Name, and Taxonomic Group) along with each amino acid (Amino Acid Name, Abreviation, Side Chain, and Molecular Weight). This can be found by scrolling over the species name or the amino acid.



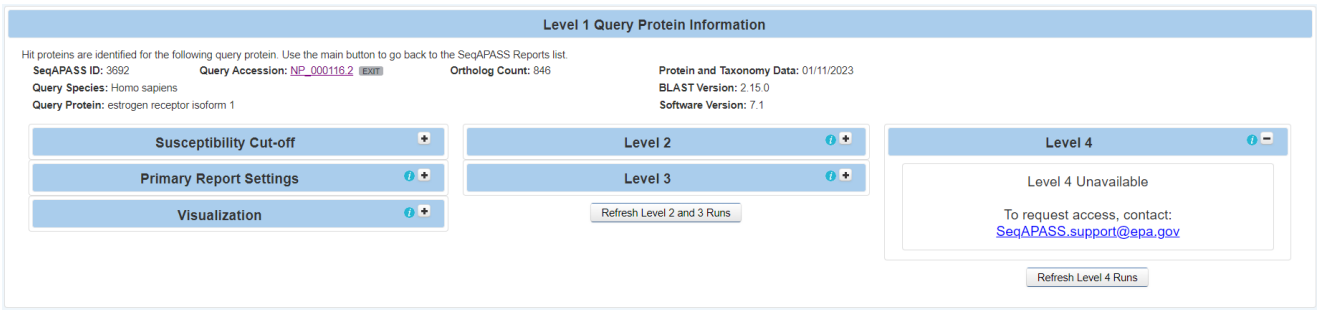


To push the designed Heat Map to the Decision Summary Report as a visualization, press the “Push Level 3 Heatmap to DS Report” button. It will then be active within the DS Report Level 3 section. To

download the Heat Map, press the "Download Heatmap…" button. The Heat Map can be downloaded as an SVG, JPG or PNG.

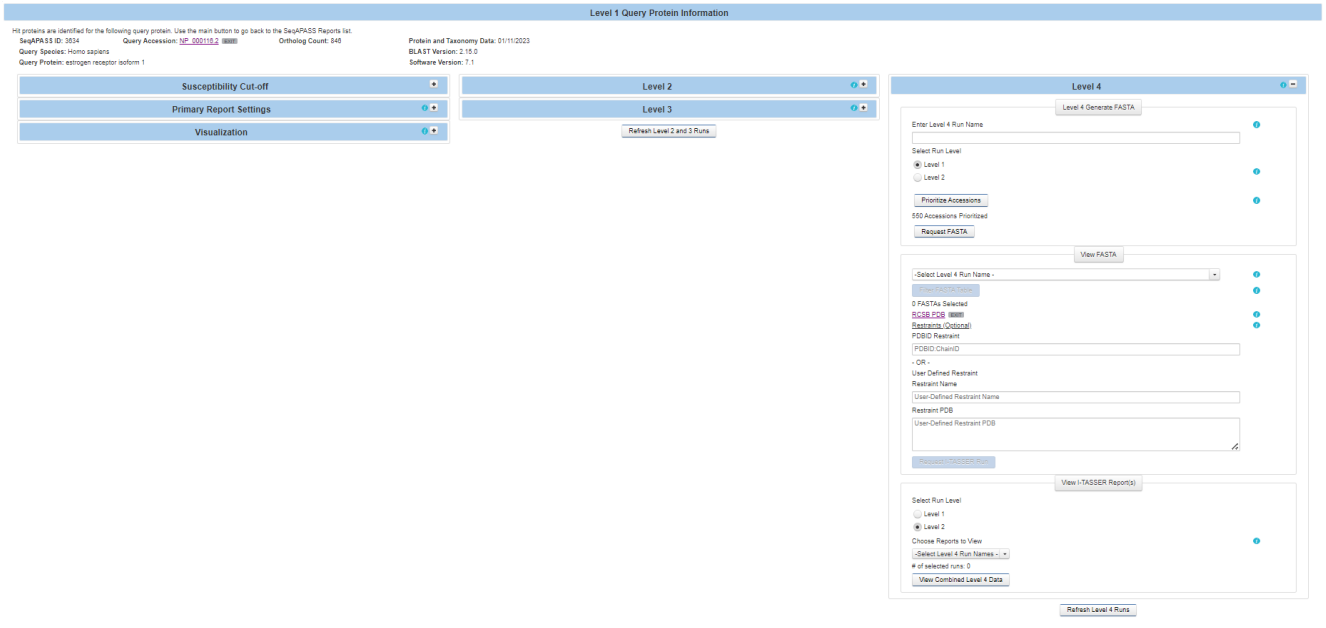
Level 4: Protein Structural Analysis

Experts in protein structural biology may access Level 4 to utilize the integrated protein structural analysis. Level 4 access can be requested through the SeqAPASS Homepage "Submit a Problem/Question" link. If a user does not have Level 4 access, expanding the Level 4 tab will display the message "Level 4 Unavailable. To request access, contact SeqAPASS.support@epa.gov".



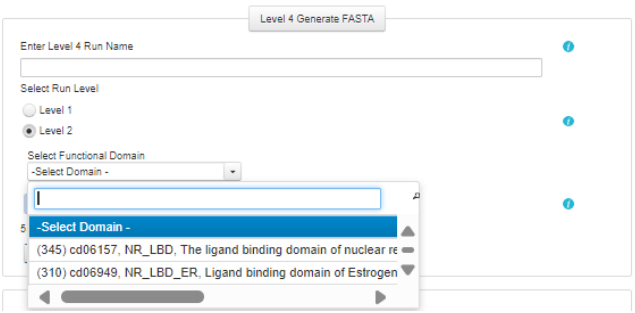
The Iterative Threading ASSEmbly Refinement (I-TASSER), AlphaFold, and TM-align tools

The I-TASSER and AlphaFold tools are capable of 3D structural generation, while TM-align analyzes multiple structures for similarity as another line of evidence toward conservation.

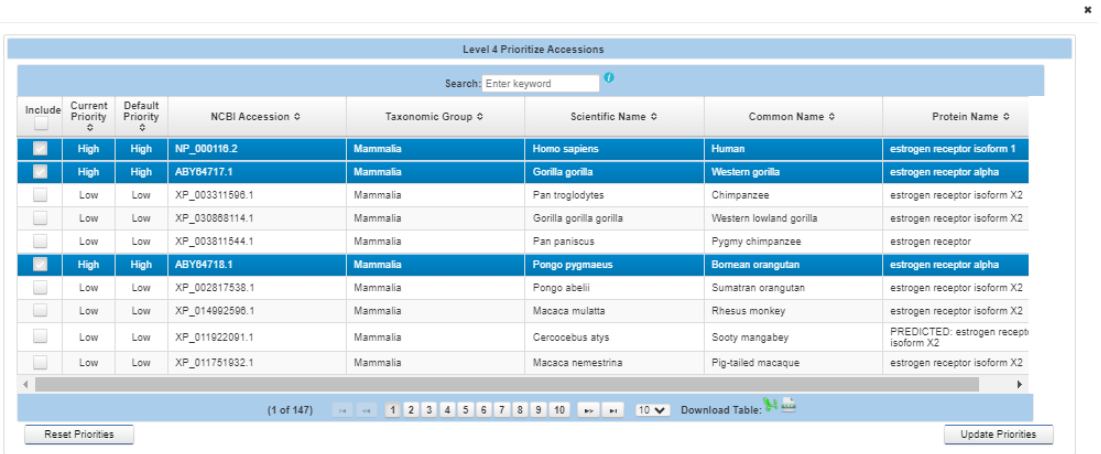


Level 4 FASTA Generation

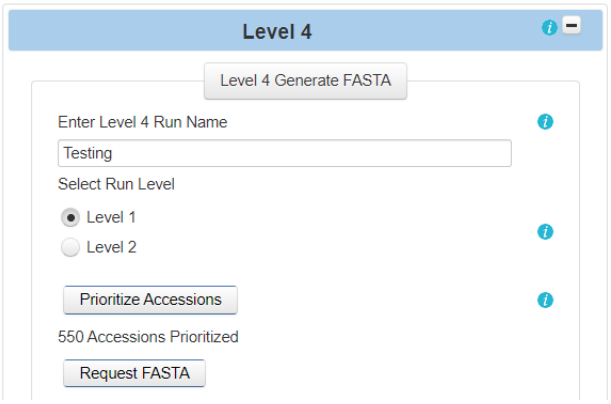
To begin the Level 4 analysis, the NCBI accessions from the Level 1 or Level 2 alignments must be converted into FASTA formatted sequences for submission to I-TASSER. Users may prioritize accessions for FASTA generation in the "Level 4 Generate FASTA" subsection by providing a user defined name for their job in the "Enter Level 4 Run Name" text box. User defined run names will automatically be updated with domain accession and PDB restraint information when those elements are selected or input. After naming the run, the user must select which sequence information they will use from either Level 1 or Level 2. The "Level 1" radio button allows users to generate predicted 3D structures from the full-length protein sequence, whereas the "Level 2" radio button allows 3D structure generation of domain-specific regions of a protein. If "Level 2" is selected, the user must use the "Select Functional Domain" dropdown to choose a specific domain for which models will be generated. Functional domains must be run first in SeqAPASS Level 2 before they can be selected in the "Select Functional Domain" dropdown in Level 4.



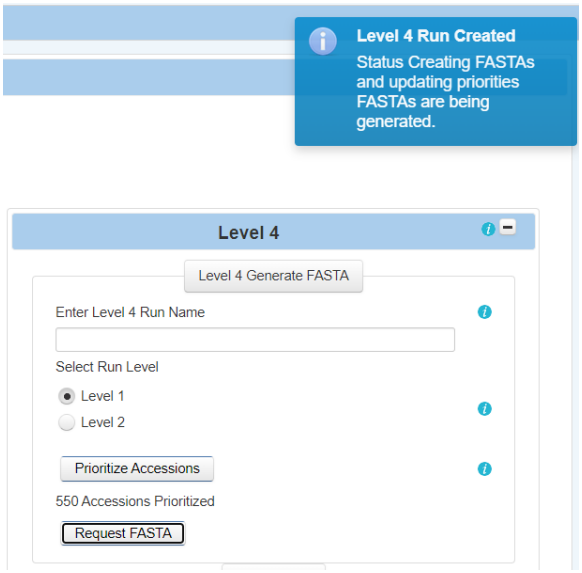
After selecting a run level, the user may prioritize accessions for FASTA generation through selecting the "Prioritize Accessions" button where a "Level 4 Prioritize Accessions" table will pop up. This table will, by default, have likely high-quality accessions preselected for FASTA generation, however, the user may add or remove any accessions available in the table using the selection checkboxes. The accessions have been pre-priorized by default to include accessions with NP_######.#, and Swiss-Prot Accessions. Sequences that are hypothetical, partial, Low quality, unnamed, unknown or XP_######.# are excluded by default. After selecting and/or removing the desired accessions, the user may click the "Update Priorities" button to confirm their prioritizations or select the "Reset Priorities" to undo any changes they have made from the default prioritizations (The "x" button may also be utilized to close the "Level 4 Prioritize Accessions" table without committing any changes).



The number of accessions selected in the "Level 4 Prioritize Accessions" table for FASTA conversion is reported below the "Prioritize Accessions" button within the "Level 4 Generate FASTA" subsection. This number will reflect any changes to the Level 4 "Prioritize Accessions" table that are submitted through the "Update Priorities" button.

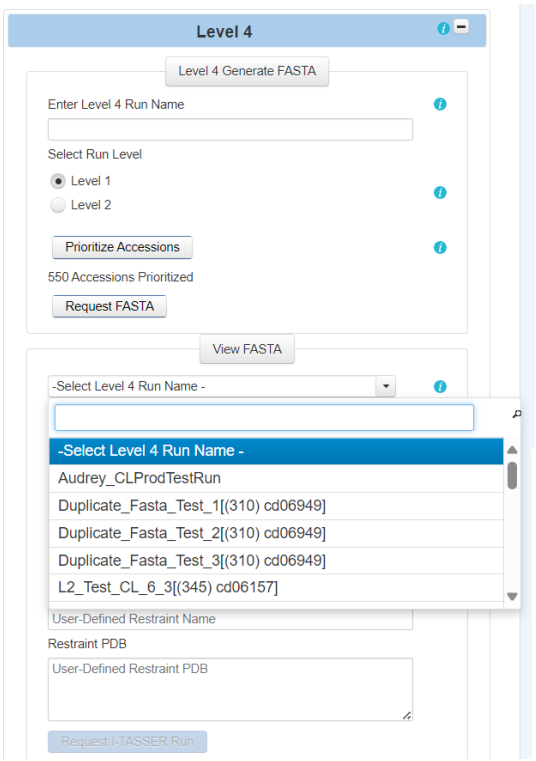


When all the desired accessions are prioritized for FASTA generation, the "Request FASTA" button may be utilized to confirm the submission. A pop-up will appear in the upper right corner to notify a successful submission.



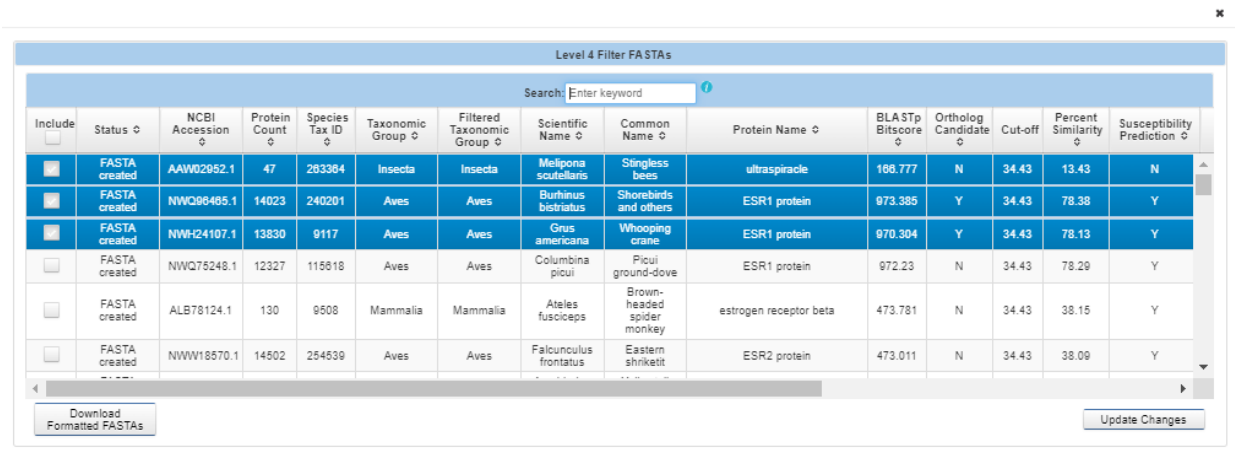
View FASTA

After submitting accessions for FASTA generation, the user may select the "Refresh Level 4 Runs" button, located beneath the "Level 4" section, to refresh the available FASTA runs for selection. The user may then select a generated FASTA report to view by clicking the "Select Level 4 Run Name" dropdown menu and selecting the desired user defined name from the completed FASTA jobs.



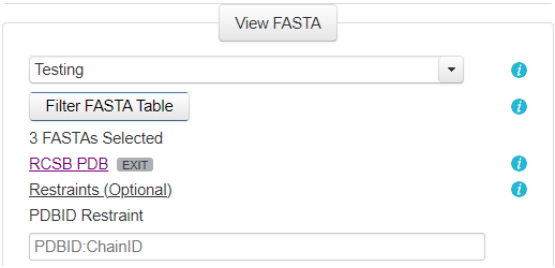
The user may then select the "Filter FASTA Table" button to bring up the "Level 4 Filter FASTAs" table. This table allows the user to add or remove generated FASTA sequences before pushing forward to I-TASSER for structure generation.

NOTE: Since I-TASSER structure generation is a timely and computationally expensive process, a maximum of **10 FASTAs** may be submitted at a time **per user**. If a user wants to generate more than 10 structures, they will need to wait until the first batch of 10 is complete before prioritizing another batch of FASTA sequences to submit to I-TASSER.

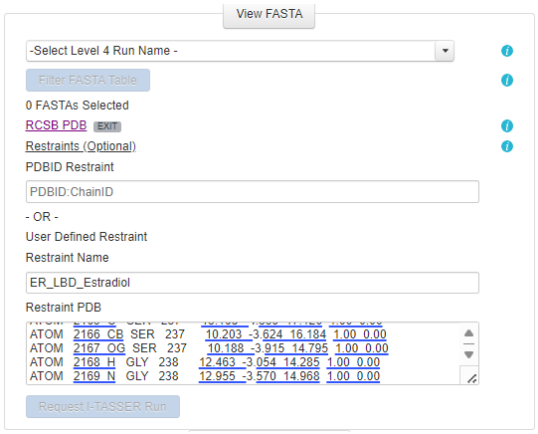


After adding and removing the desired FASTA accessions for I-TASSER, the user may select the "Update Changes" button to confirm their changes, or the "x" button to cancel their changes. After confirming any changes, the "x FASTAs Selected" text will display the current number of selected

FASTAs for I-TASSERs submission. The user can also download all FASTAs listed in the table with the "Download Formatted FASTAs" button.



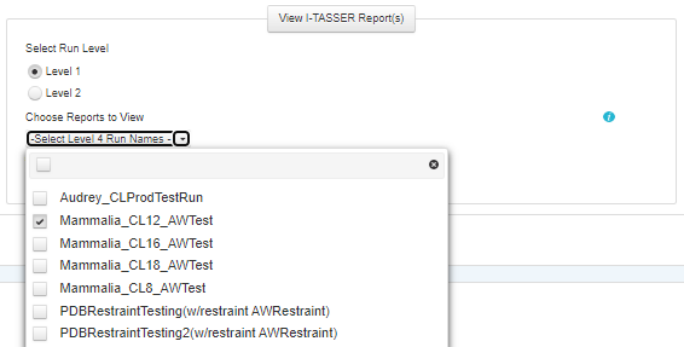
The user may also choose a structural restraint for their I-TASSER structure generation by typing the PDBID:ChainID into the textbox beneath the "Choose I-TASSER Restraint" text. The I-TASSER Restraint serves as a reference for I-TASSER's structural generation. Alternatively, a user may define their own structural restraint for structural prediction, using a PDB file of their choosing. To do so, users must first define a unique name in the "Restraint Name" text box. Users should then open the PDB file of the restraint in a txt editor, select the full text representation of the PDB, and paste the text into the "Restraint PDB" text box.



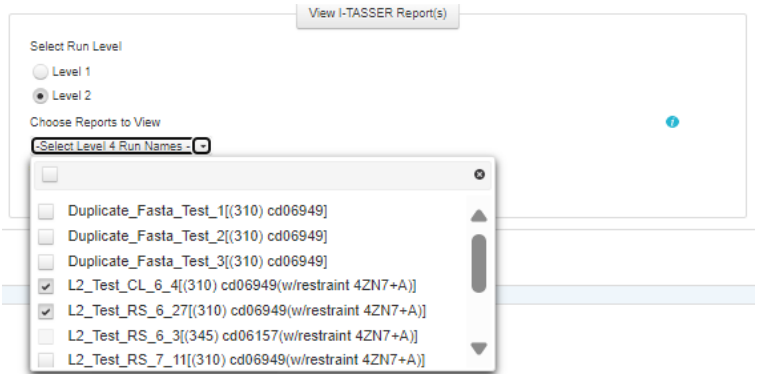
When all desired FASTAs are selected, click the "Request I-TASSER Run" button to submit the selected FASTAs to I-TASSER.

View I-TASSER Report

After at least one job has completed from those submitted to I-TASSER, the user may select the "Refresh Level 4 Runs" button to reload the page with any available user defined report names. The user must then select which structures and corresponding metrics to view using the radio buttons for either Level 1 or Level 2 completed jobs. Users may click the "Select Level 4 Run Names" dropdown menu and select their desired jobs for viewing. Multiple runs may be selected and combined.



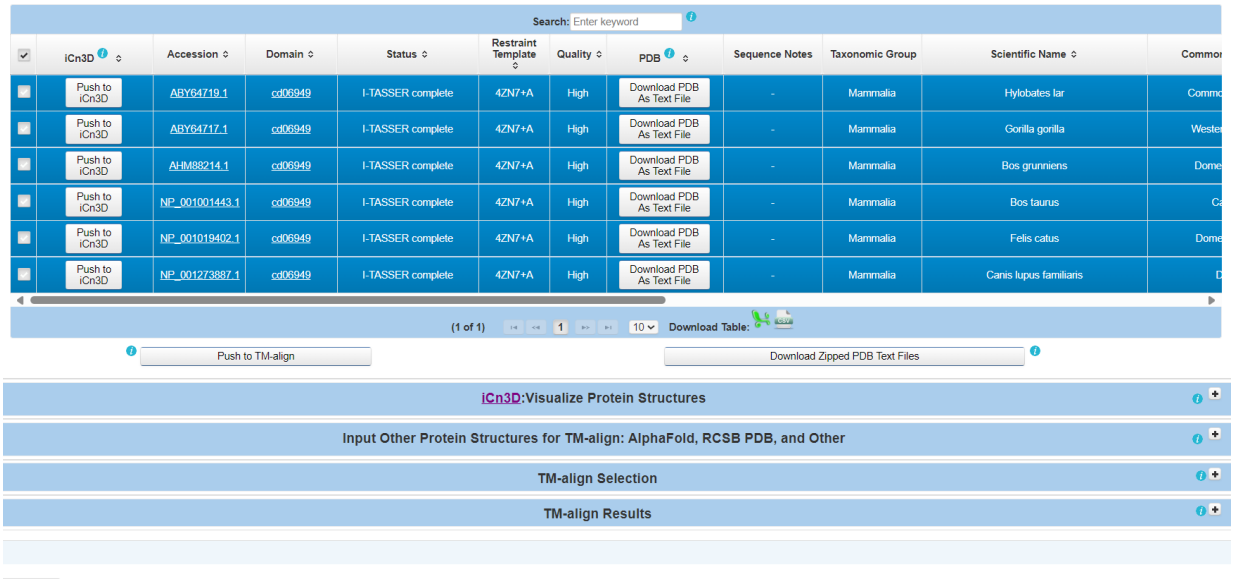
If Level 2 jobs are being selected, only those from the same domain can be combined. Once multiple reports using the same domain are selected, the top check box may be used to deselect all runs so that a different domain can be chosen.



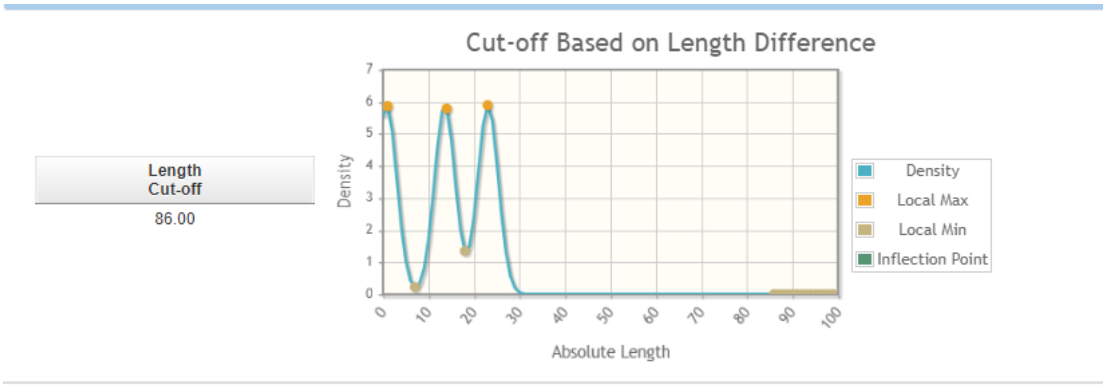
Since there is a maximum of 10 I-TASSER jobs per user, jobs of more than 10 FASTAs will need to be split into multiple jobs and combined later for viewing.

Level 4 Page

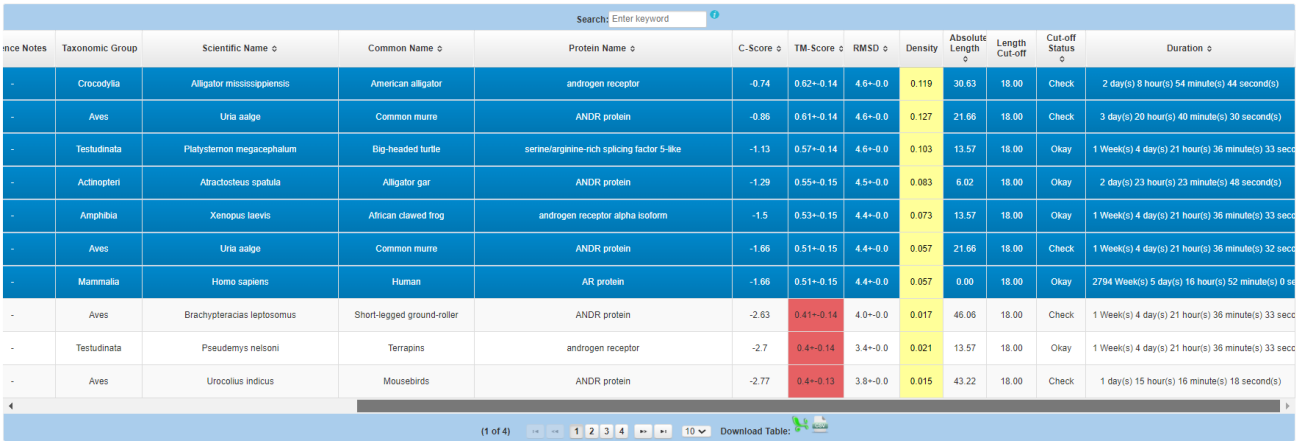
The Level 4 Data Page contains all available metrics from I-TASSER on the selected run including the C-Score, TM-Score, RMSD value, Density, Absolute Length, Length Cut-Off, Cut-Off Status, and Duration. The table also reports taxonomic information associated with each accession, including the taxonomic group, scientific name, and common name.



The Level 4 Report page displays a length density plot providing the cut-off based on the absolute length difference between each hit structure and the query structure (defined by the Level 1 query sequence). The Length Cut-Off is determined by the second local minimum following the global maximum among the absolute lengths.



This page contains the completed I-TASSER job information, alongside a density plot displaying the length cut-offs and absolute lengths of the proteins. The “I-TASSER Result and PDB Selection” table displays all accessions with completed I-TASSER jobs. Each row of the data table contains the taxonomic group, scientific name, common name, protein name, and domain accession (if applicable), as well as I-TASSER data including the C-Score, TM-Score, RMSD value, Density, Absolute length, Length Cut-Off, Cut-Off Status, and I-TASSER Status.



Search: Enter keyword

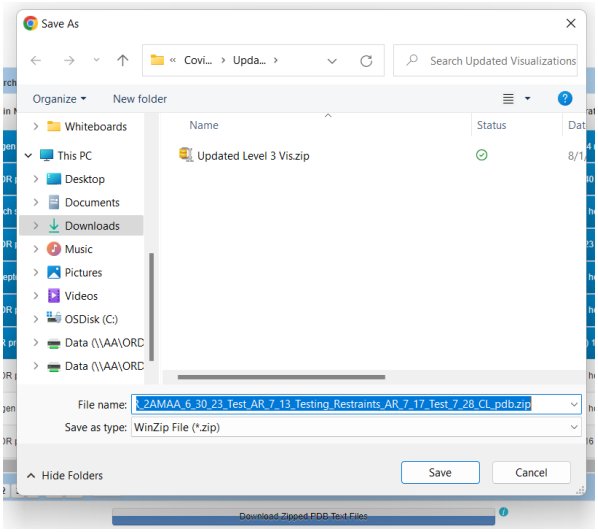
nce Notes	Taxonomic Group	Scientific Name	Common Name	Protein Name	C-Score	TM-Score	RMSD	Density	Absolute Length	Length Cut-off	Cut-off Status	Duration
-	Crocodylia	Alligator mississippiensis	American alligator	androgen receptor	-0.74	0.62+-0.14	4.6+-0.0	0.119	30.63	18.00	Check	2 day(s) 8 hour(s) 54 minute(s) 44 second(s)
-	Aves	Uria aalge	Common murre	ANDR protein	-0.86	0.61+-0.14	4.6+-0.0	0.127	21.66	18.00	Check	3 day(s) 20 hour(s) 40 minute(s) 30 second(s)
-	Testudinata	Platysternon megacephalum	Big-headed turtle	serine/arginine-rich splicing factor 5-like	-1.13	0.57+-0.14	4.6+-0.0	0.103	13.57	18.00	Okay	1 Week(s) 4 day(s) 21 hour(s) 36 minute(s) 33 secc
-	Actinopteri	Atractosteus spatula	Alligator gar	ANDR protein	-1.29	0.55+-0.15	4.5+-0.0	0.083	6.02	18.00	Okay	2 day(s) 23 hour(s) 23 minute(s) 48 second(s)
-	Amphibia	Xenopus laevis	African clawed frog	androgen receptor alpha isoform	-1.5	0.53+-0.15	4.4+-0.0	0.073	13.57	18.00	Okay	1 Week(s) 4 day(s) 21 hour(s) 36 minute(s) 33 secc
-	Aves	Uria aalge	Common murre	ANDR protein	-1.66	0.51+-0.15	4.4+-0.0	0.057	21.66	18.00	Check	1 Week(s) 4 day(s) 21 hour(s) 36 minute(s) 32 secc
-	Mammalia	Homo sapiens	Human	AR protein	-1.66	0.51+-0.15	4.4+-0.0	0.057	0.00	18.00	Okay	2794 Week(s) 5 day(s) 16 hour(s) 52 minute(s) 0 se
-	Aves	Brachypteracias leptosomus	Short-legged ground-roller	ANDR protein	-2.63	0.41+-0.14	4.0+-0.0	0.017	46.06	18.00	Check	1 Week(s) 4 day(s) 21 hour(s) 36 minute(s) 33 secc
-	Testudinata	Pseudemys nelsoni	Terrapins	androgen receptor	-2.7	0.4+-0.14	3.4+-0.0	0.021	13.57	18.00	Okay	1 Week(s) 4 day(s) 21 hour(s) 36 minute(s) 33 secc
-	Aves	Urocolius indicus	Mousebirds	ANDR protein	-2.77	0.4+-0.13	3.8+-0.0	0.015	43.22	18.00	Check	1 day(s) 15 hour(s) 16 minute(s) 18 second(s)

(1 of 4) 1 2 3 4 10 Download Table:

If the Cut-off Status for an I-TASSER result is "Check", then it is recommended that the user use a structure visualization tool, such as the embedded iCn3D tool, to determine if the structure needs modification before further analysis. The user is notified to "Check" when the absolute difference between the query and hit lengths is greater than the length cutoff value. In this instance, the I-TASSER generated structure may be represented differently than the query structure (e.g., focusing on a domain as opposed to the full protein structure).

From this table, the user may select desired structures to analyze with TM-align. When the desired accessions are selected using the check boxes in the left most column of the table, click the "Push to TM-align" button to populate the "TM-align" Selection table.

The I-TASSER generated structures are downloadable as a PDB formatted files in the table. The user may download all generated protein structures as a Zip file through the "Download Zipped PDB Text Files" button.

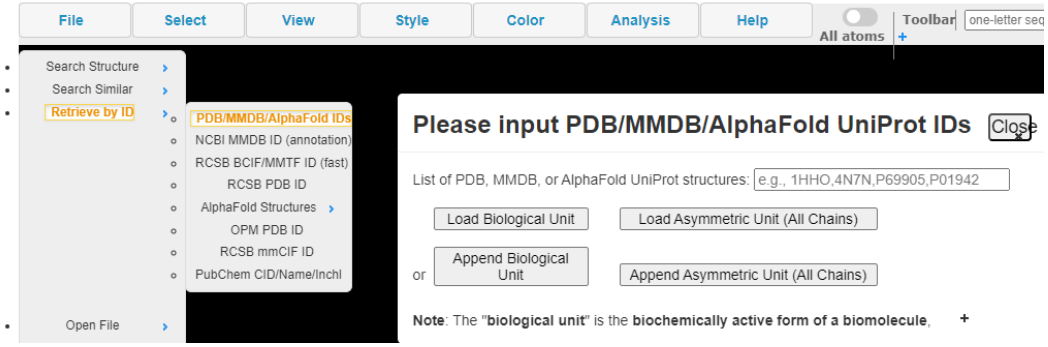


These Zip Files will contain all generated PDBs from the I-TASSER output in PDB format and will be named by the protein accession.

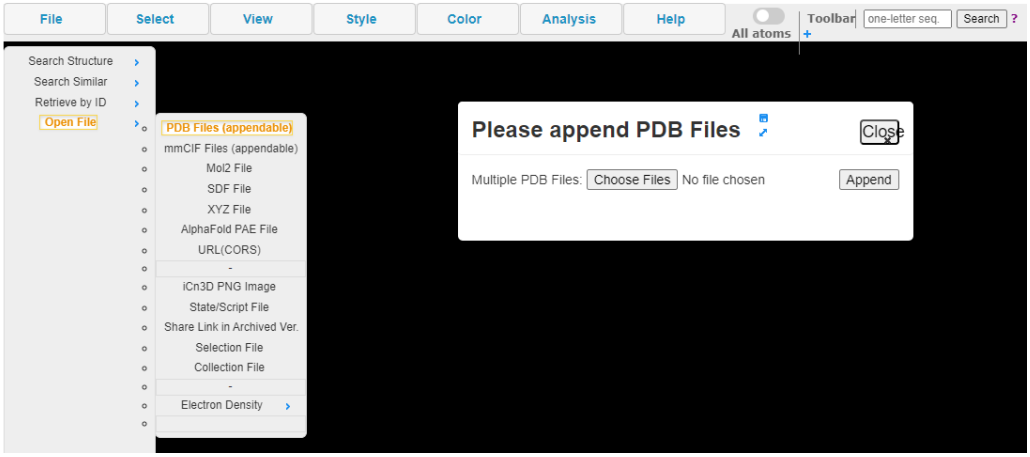
iCn3D: Visualize Protein Structures

Users may push PDB files from the "I-TASSER Results & TM-align Selection" table into the iCn3D visualization section using the "Push to iCn3D" button. Structures will automatically load into the iCn3D structure visualization box, which automatically expands.

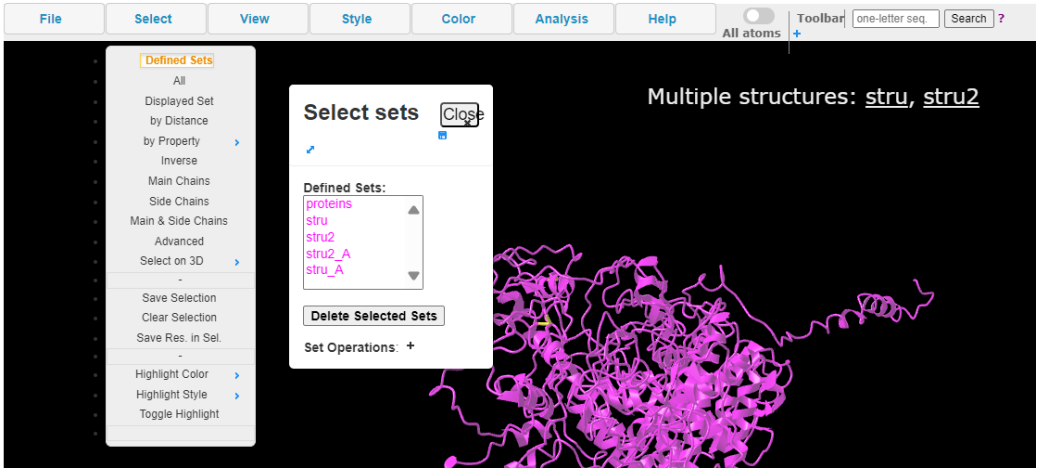
Other protein structures can be loaded for comparison from the RCSB database by clicking "File" → "Retrieve by ID" → "PDB/MMDB/AlphaFold IDs".



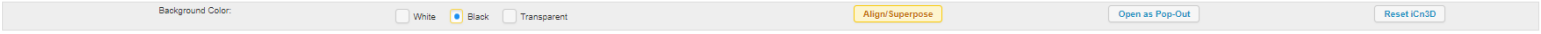
PDB files can be loaded from the user's computer via "File" → "Open File" → "PDB Files (appendable)".



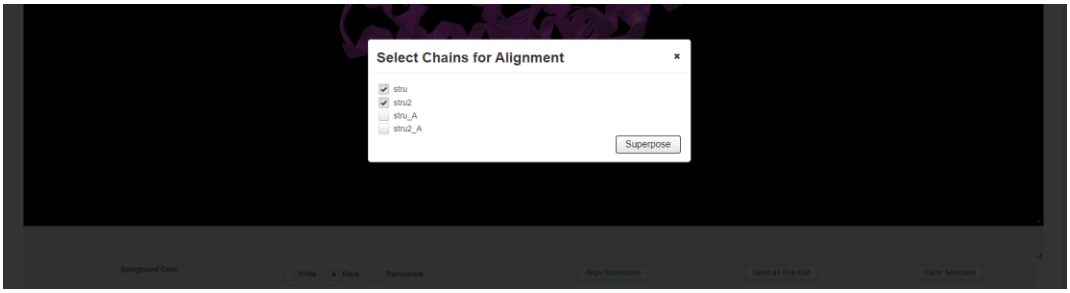
Once multiple structures have been loaded into iCn3D, users can select specific proteins for viewing or analysis by clicking "Select" → "Defined Sets" to view a defined sets menu.

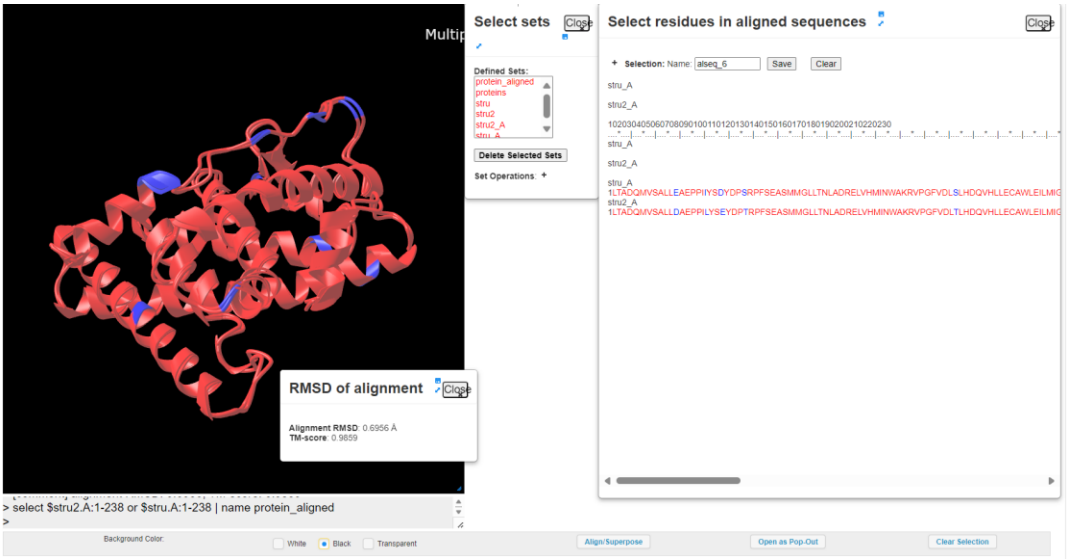


If multiple structures have been loaded, they can be superposed using the "Align/Superpose" button found beneath the iCn3D visualization.

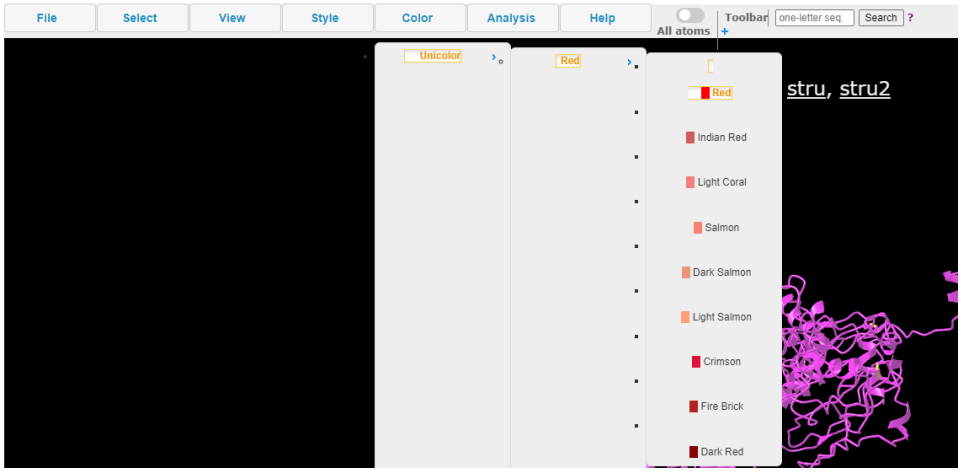


This opens a dialogue box in which users can select all proteins or defined sets desired for visual superposition. This will also generate a side panel showing a 2D alignment of annotated protein sequences.

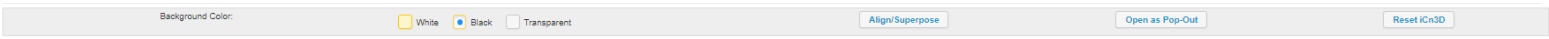




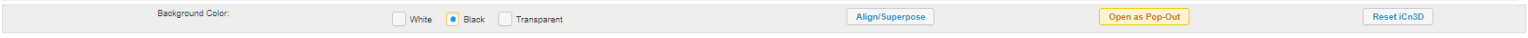
Users can alter the color of proteins using the "Color" menu in iCn3D.

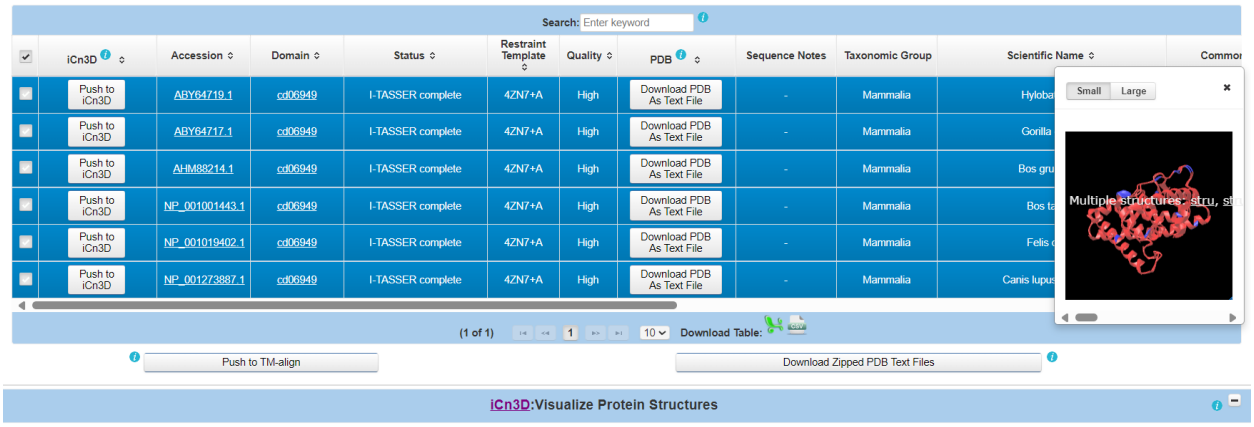


Users can also change the background color of the iCn3D window between white, black, and clear using the toggle buttons appearing beneath the window.



Additionally, the iCn3D window can be resized using the "Open as Pop-Out" button into a floating pop-out that can be moved around the screen. To continue modifying structures, users must click the "X" at the top right of the pop-out and return to the full iCn3D view.



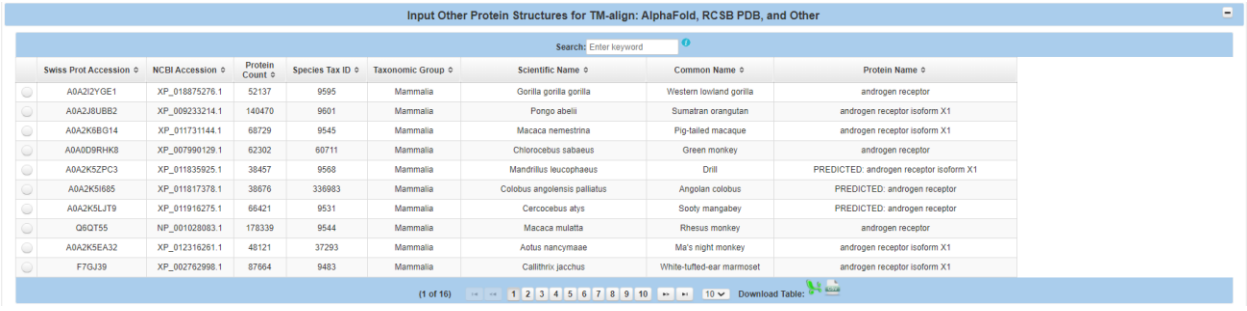


When users wish to visualize other proteins and/or remove the currently viewed proteins, they can click the "Reset iCn3D" button to remove all proteins currently held in the iCn3D window and revert iCn3D to a blank window.

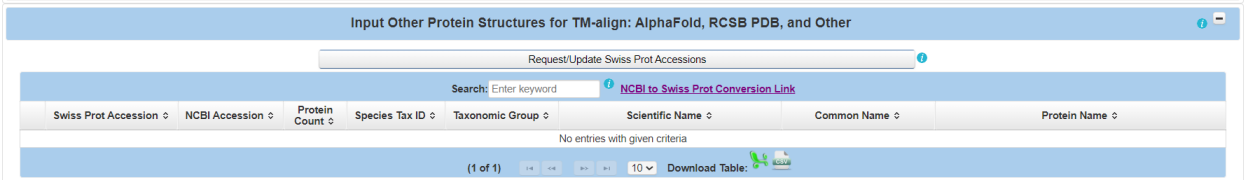
Users can also type in the command box beneath the iCn3D window to enter command shortcuts for customization and analysis. Further details on the capabilities of iCn3D, as well as lists of command prompts, can be found in the "Help" tab of the iCn3D window menu.

Input Other Protein Structures for TM-align: AlphaFold, RCSB PDB, and Other

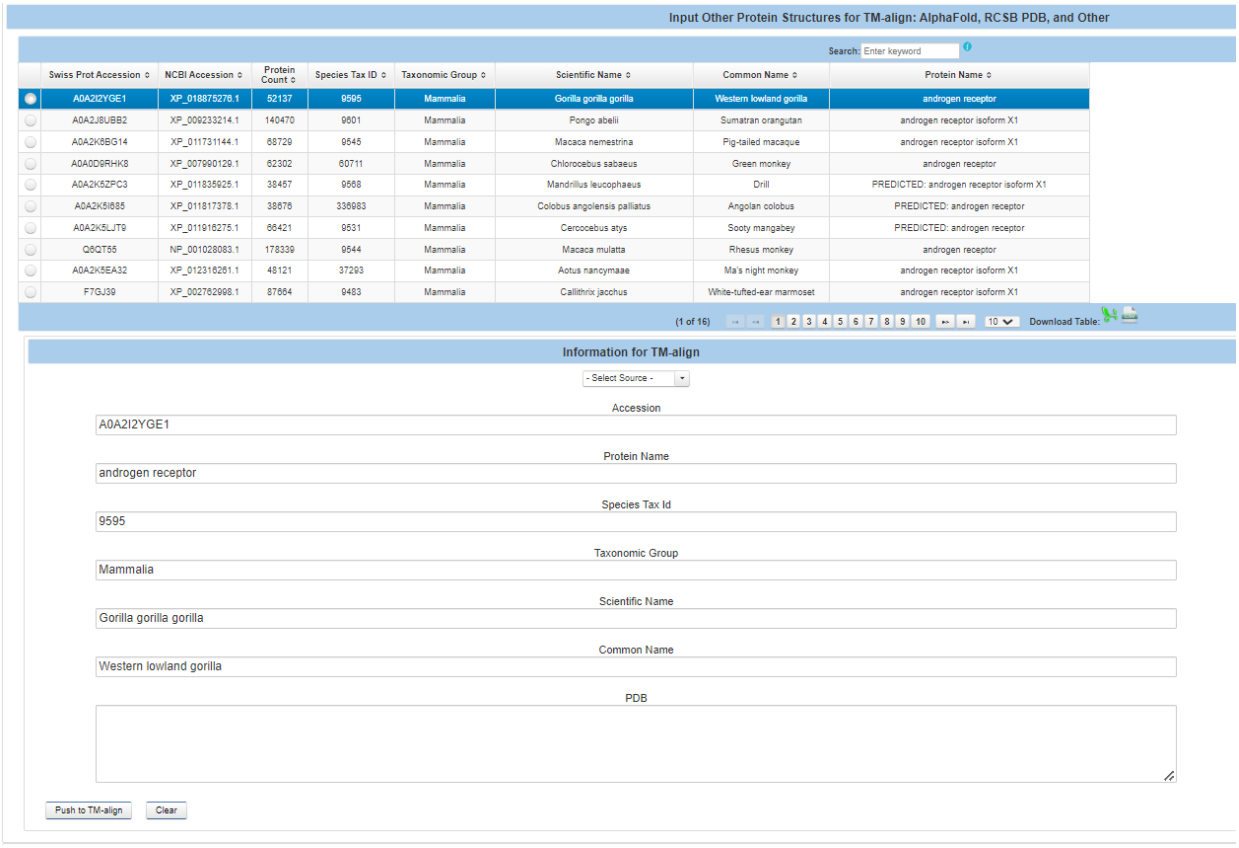
Users may bring in externally generated PDBs with the optional Input Other Protein Structures for TM-align feature. This section consists of a table containing Swiss Prot accessions associated with the Level 1 Full Report output as well as a separate section containing multiple text boxes for submitting PDB information for integration of externally generated structures in TM-align.



To access the table of Swiss Prot accessions, the user must first click the "Request/Update Swiss Prot Accessions" button. This updates the table below with NCBI to Swiss Prot conversions based on the current Level 1 Full Report. This process may take some time to complete depending on the number of accessions in the Level 1 Full Report.



Once the table has been populated, the user may select desired Swiss Prot accessions and access the AlphaFold website through the provided link. By selecting a Swiss Prot accession, the table containing text boxes will automatically populate all textboxes except the "PDB" text box.



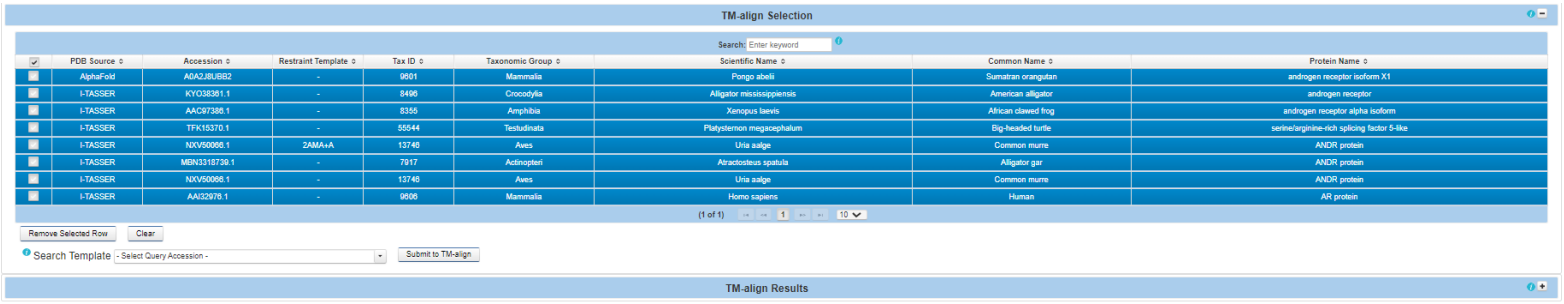
The 'PDB" text box is for the user to copy and paste their externally generated PDB. To do this, the PDB file must be opened in a txt editor and the user will need to copy the full text of the file to their clipboard and paste the text into the PDB text box.

TM-align

TM-align is an algorithm for sequence independent protein structure comparisons (https://zhanggroup.org/TM-align/). TM-align compares only two structures at a time, the query structure and the template structure. It outputs a score (the TM-score) representing the similarity of the two structures as a value between 0 and 1 where 1 represents a perfect match between the aligned structures.

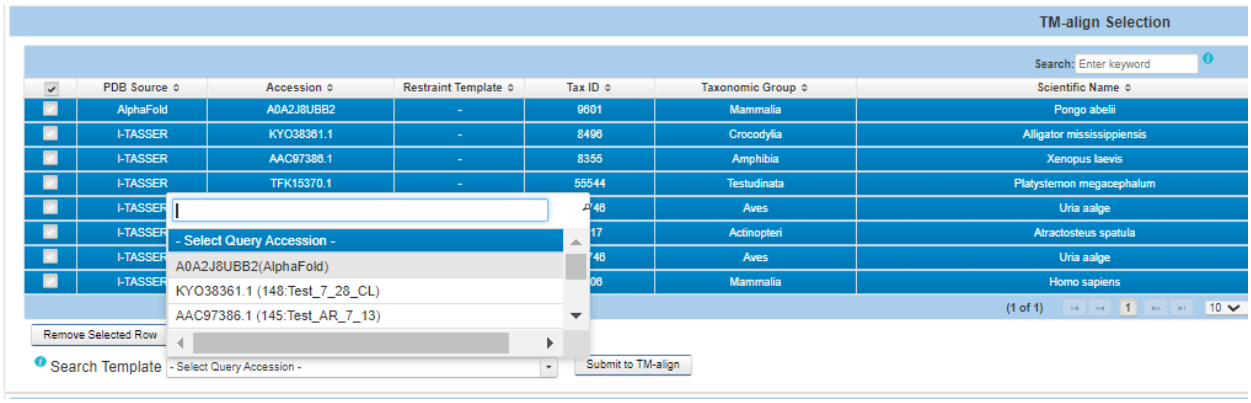
Users may select accessions in the I-TASSER table after they have completed generating PDB files using the checkboxes and select the "Push to TM-align" button to send the selected PDBs to a queuing table found in the TM-align Selection section of the Level 4 data page before submission. Users may also add

externally generated PDBs to the queuing table through the "Input Other Protein Structures for TM-align: AlphaFold, RCSB PDB, and Other" section.



The user may remove singular PDBs from the queuing table by selecting the checkbox button in the far-left column of the accession's row and selecting the "Remove Selected Row" button. The user may also clear all queued accessions through the "Clear" button.

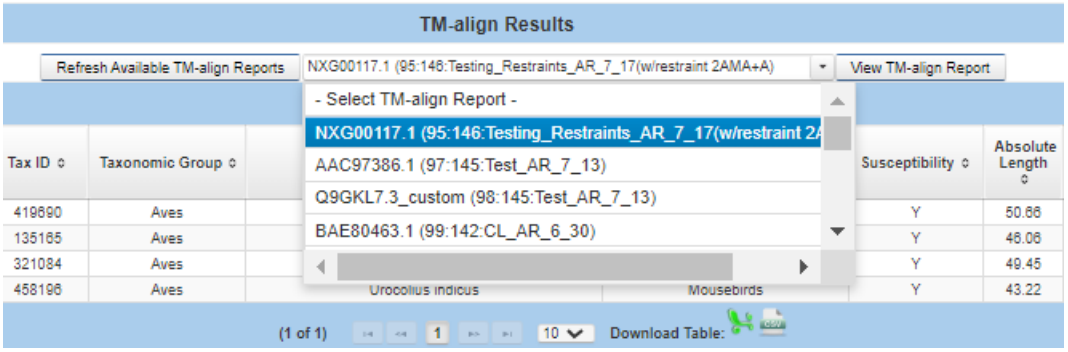
Once the user has added all the desired PDB structure files to the queuing table, they may select a template accession. This is the structure all other structures will be compared to in TM-align. The user may select any PDB generated by I-TASSER or integrated into the TM-align table from external sources as their template.



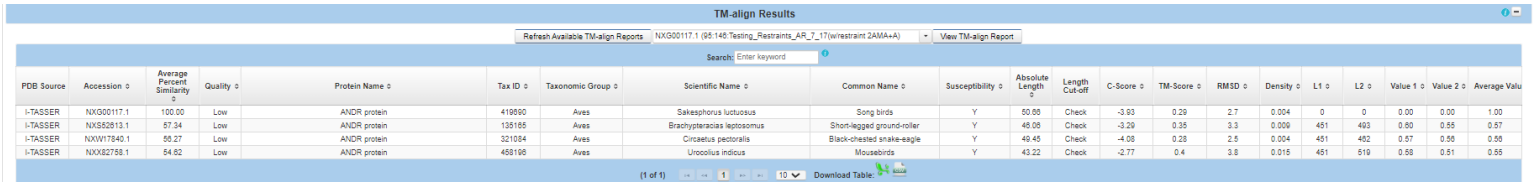
The templates are uniquely named by their accession (I-TASSER SeqAPASS Run Id: User defined name (w/restraint text autogenerated). Once all desired PDBs are queued and a template chosen, the user may click the "Submit to TM-align" button to submit their PDBs for structural alignment.

TM-align Results

To view the results for TM-align, select the dropdown menu within the "TM-align Results" table. This dropdown menu will contain all processed TM-align runs pertaining to the current Level 4 report. After selecting the desired run, select the "View TM-align Report" button to bring up the results table.



The "TM-align Results" table reports the metrics generated by TM-align structural alignment of the chosen PDBs. These results include the L1 value which is the length of the selected PDB and the L2 value, which is the length of the template PDB. The table also reports the I-TASSER metrics reported in the previous "I-TASSER Results" table as well as the TM-align metrics which include "Value 1", "Value 2", and "Average Value". "Value 1" reports the alignment of the template structure to the hit structure, while "Value 2" reports the alignment of the hit structure to the template. The "Average Value" then reports and average of both "Value 1" and "Value 2". Since accessions must go through multiple filters during the Level 1, Level 2, Level 3, and Level 4 analysis, the expectation is that the accessions used for PDB generation are of high quality and relevance. The susceptibility call-out for the "TM-align Results" table is therefore considered "Yes" by default, but experienced users should make their own determination of susceptibility after the alignment of the generated Level 4 structures.



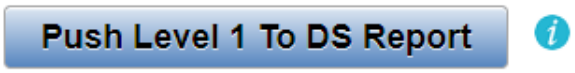
The "TM-align Results" table may be downloaded separately as either an .excel or .csv file through the "Download Table" icons.

Decision Summary Report

The "Decision Summary (DS) Report" is a feature that gives the user options to design a single output page to concisely view results from all Levels of the SeqAPASS evaluation for completed jobs. The output is customizable to include visualizations and susceptibility predictions that can be downloaded in a PDF format. The "DS Report" page becomes activate when the user utilizes any of the "Push to DS Report" buttons located throughout the toolset. The "DS Report" page will contain a maximum of one Level 1 output (and visualization) and one Level 3 output (and visualization) but can contain multiple Level 2 domain outputs (and their respective visualizations).

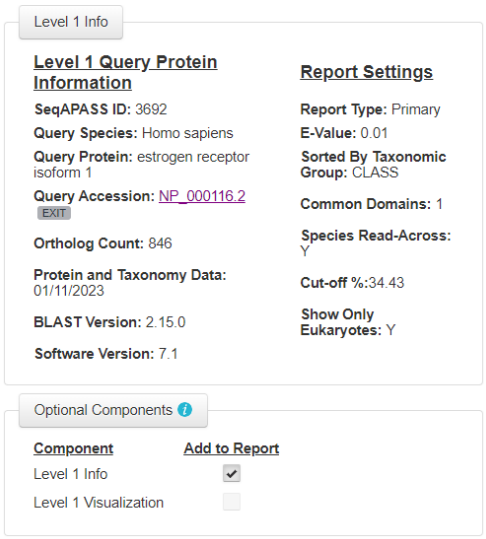
To push results from any Level to the DS Report, the user must press the "Push Level # To DS Report" button. The "DS Report" button will become active for the user to view the report settings. The DS Report can be updated as the user changes settings in Level 1, Level 2, and Level 3 (Adding or removing

amino acids) but the user must push the updated report to the DS Report again using the "Push Level # To DS Report" button (There will be a notification next to the button if settings have been updated to remind the user to push the report). If the user chooses to change to a different SeqAPASS job (e.g., a different protein accession), the "DS Report" button will become inactive, and the user must push the data from the new job to the DS Report as described previously.



Level 1 of the Decision Summary Report

Upon clicking the "DS Report" button, the user is brought to a new page that will contain the "Level 1 Report" section of the DS Report which will show all the pertinent information for the query protein and report settings that were pushed to the report. The user can also include the Level 1 visualization in the DS Report by going to the "Level 1 Visualization" page and clicking "Push to Boxplot to DS Report". The default visualization or a user customized visualization will then be inserted in the downloadable DS Report PDF once the radio button is selected.



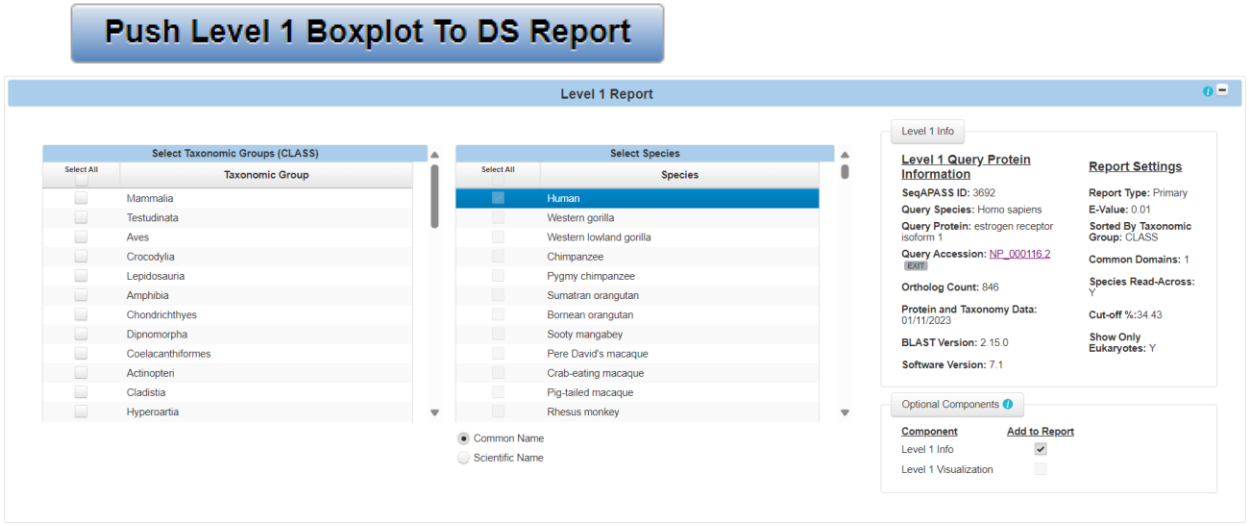
Once the user is satisfied with the data that has been pushed to the DS Report, the "DS Report" button will bring the user to the "Level 1 Report" section which gives the user customizable options. In the "Level 1 Report" section, there is a series of checkboxes in the "Select Taxonomic Groups (CLASS)" box. Here the user can select which taxonomic group(s) they would like to select and display in the DS Report. Upon selecting the taxonomic group(s), the user can then customize the report in the "Select Species" box, by selecting the checkbox next to the species for which the user would like data from Level 1 displayed in the "Final Decision Summary Report" table at the bottom of the page. The template species will always be selected and cannot be deselected. Specie(s) will be active only when at least one taxonomic group is selected in the "Select Taxonomic Groups (CLASS)" box. Level 1 results for those species selected from the "Select Species" box will be integrated in the "Final Decision Summary Report" table at the bottom of the page (Note: if the user does not push a Level 1 job to the "DS Report" page, there will be no information in that section).

Level 1 Info

The Level 1 information section becomes present when either a Level 1 report or a Level 1 visualization is pushed to the DS Report. The information contained in the section includes the "Level 1 Query Protein Information" (i.e., SeqAPASS ID, Query Species, Protein, and Accession, Ortholog Count, Protein and Taxonomy Data, Blast Version and Software Version.) as well as the "Report Settings" (i.e., Report Type, E-Value, Sorted By Taxonomic Group, Common Domains, Species Read-Across, Cut-Off, and Show Only Eukaryotes.) and finally the "Optional Components" section which contains the option to include the "Level 1 Visualization" to the report.

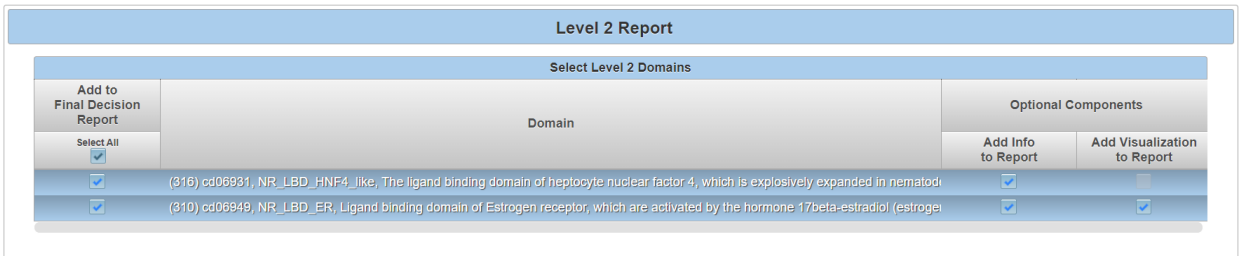
Including Visualizations in DS Reports

The user can also include the "Level 1 Visualization" by going to the visualization page and either pushing the default visualization or a user modified visualization which will then be attached in the downloaded PDF once the radio button is selected. In the scroll downs, the template species will always be selected and cannot be deselected. Specie(s) will be not active until a taxonomic group box is selected. Once that occurs, those respective species will become active and can be deselected individually or by the select all function. Those species selected will become active in the "Final Decision Summary Report" table at the bottom of the page (Note: if a user pushed only a boxplot to the DS Report, then only the "Level 1 Info" and the "Optional Components" will be active).



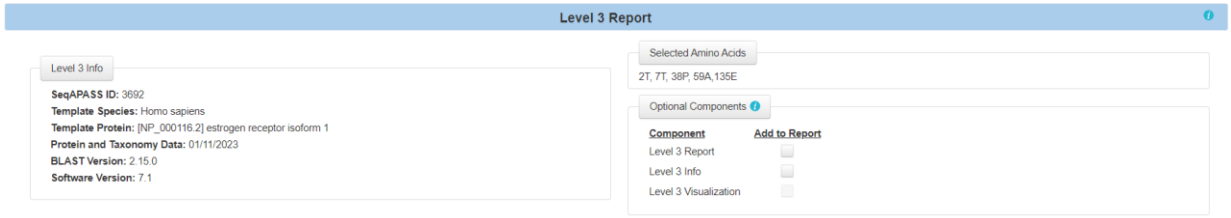
Level 2 of DS Report

The Level 2 section of the DS Report contains all the domains that have been pushed to the report. There can be multiple domains present in the section once they have been run and pushed individually to the report. The user can also include each respective "Level 2 Visualization" by going to the visualization page and either pushing the default visualization or a user modified visualization which will then have the option to be attached in the downloaded PDF. Once a domain is selected, it will appear in the "Final Decision Summary Report" table at the bottom of the page (Notes: if the user does not push a Level 2 run to the DS Report page, there will be no information in that section. If a visualization is pushed to the DS Report before a Level 2 report, the domain will be present along with the "Add Visualization to Report" button being active.).



Level 3 of DS Report

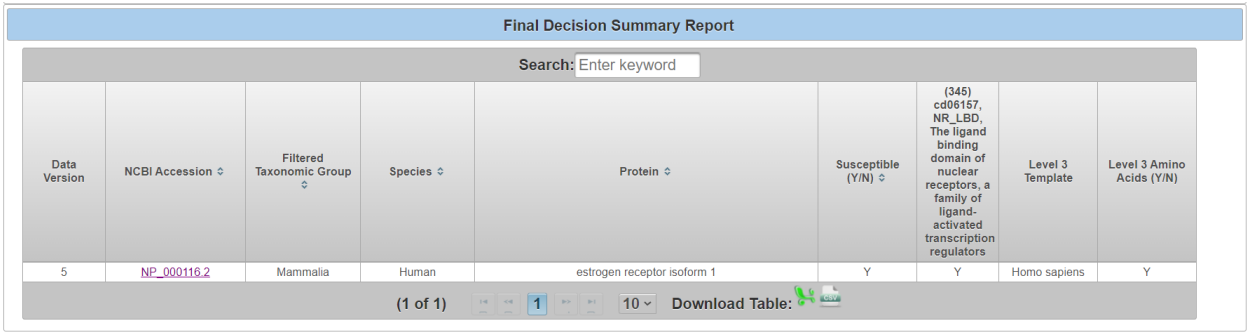
The Level 3 section of the DS Report contains all the information for the query protein and report settings that were pushed to the report. It also contains the amino acids that were updated in the report and pushed over. New amino acids will need to be pushed over to the DS Report. The Yes (Y) or No (N) susceptibility will be displayed in the "Final Decision Summary Report" table. The user can also include the "Level 3 Visualization" by going to the visualization page pushing a user modified visualization which will then have the option to be attached in the downloaded PDF. (Notes: if the user does not push a Level 3 run to the DS Report page, there will be no information in that section. Also, if a "Level 3 Visualization" (Heat Map) is pushed before a Level 3 report, the "Level 3 Info" will be populated with that respective run's information.)



Final Decision Summary Report Table

The "Final Decision Summary Report" table contains the important data and susceptibility predictions for each level run, for all the species selected in the Level one section. The table takes the susceptibility prediction for each run and easily displays the results for a quick interpretation. The complete table can be either saved as an excel spreadsheet or .csv file. It will also be added into the PDF when downloaded.

Each selected specie(s) will have its own respective row which contains the information that has been pushed to the "Final Decision Summary Report" table. The columns will show the Data Version, NCBI Accession, Filtered Taxonomic Group, Species, Protein Name, Level 1 Susceptibility Prediction as Yes (Y) or No (N), Level 2 Common Domain(s) Name and respective Susceptibility Prediction as Yes (Y) or No (N), Level 3 Template Species, and Level 3 Amino Acid Susceptibility Prediction as Yes (Y) or No (N). (A few things to note: if there are multiple domains pushed to the "Final Decision Summary Report" table, each domain will have their own column. Also, for species to have either a Yes (Y) or No (N) susceptibility prediction in the table, they must be pushed to the report from the Level 3 run as well as selected in the Level 1 taxonomic groups/species selection. If a species was not included in the Level 3 report that was pushed but is included in the "Final Decision Summary Report" table, they will receive a NA for their Level 3 susceptibility prediction.)



Download DS Report as PDF

To capture all the data pushed to the DS Report as a PDF, press the "Download DS Report" button. The DS Report PDF will match the data on the DS Report page and will include the visualizations if selected by the user. The information for each Level that is pushed to the downloaded DS Report PDF include all the Query Protein Information for that respective protein, domain(s), and template protein. (Note: Once the PDF is created and the DS Report page has been updated, the user must redownload the PDF to have the most up to date version of the page.)

Level 1 of DS Report PDF

The Level 1 section of the DS Report PDF will contain all the "Level 1 Query Protein Information" along with the Level 1 "Report Settings" for that respective protein's run. This information will not be present if no Level 1 run information or Level 1 visualization is pushed to the DS Report PDF.

Level 1

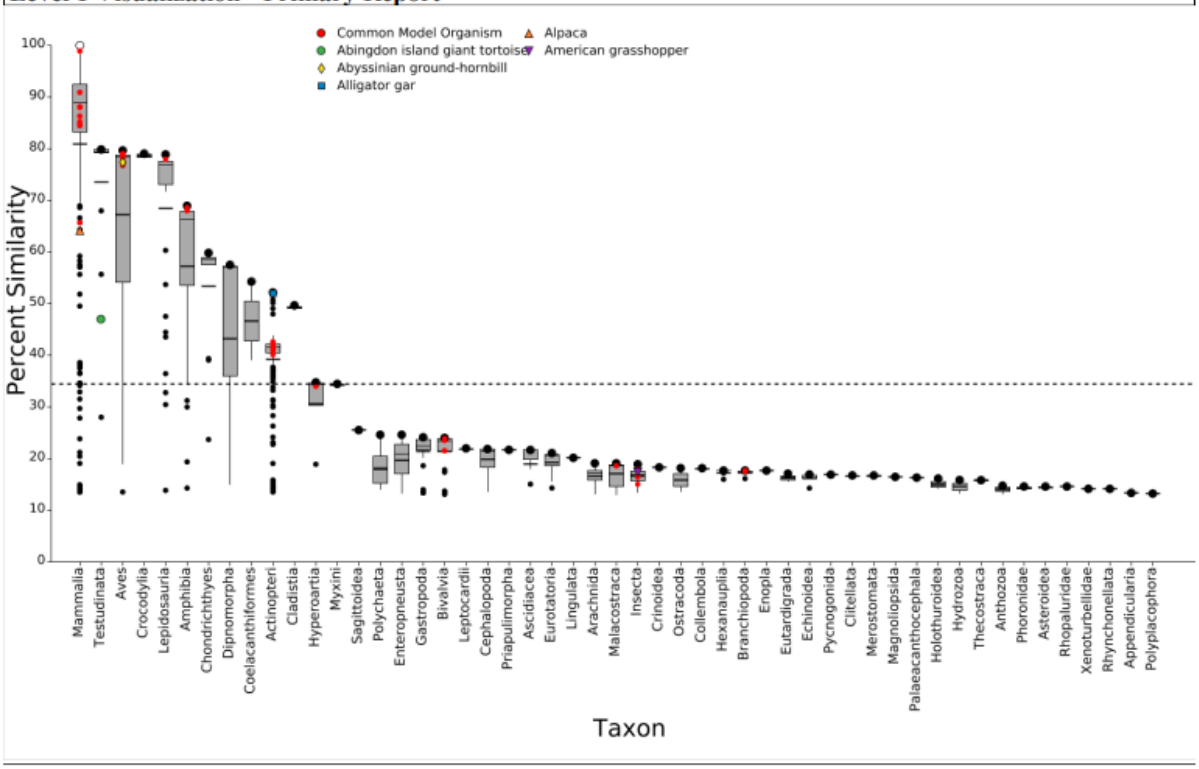
Level 1 Query Protein Information

SeqAPASS ID: 3692
Query Species: Homo sapiens
Query Protein: estrogen receptor isoform 1
Query Accession: NP_000116.2
Ortholog Count: 846
Protein and Taxonomy Data: 01/11/2023
BLAST Version: 2.15.0
Software Version: 7.1

Report Settings

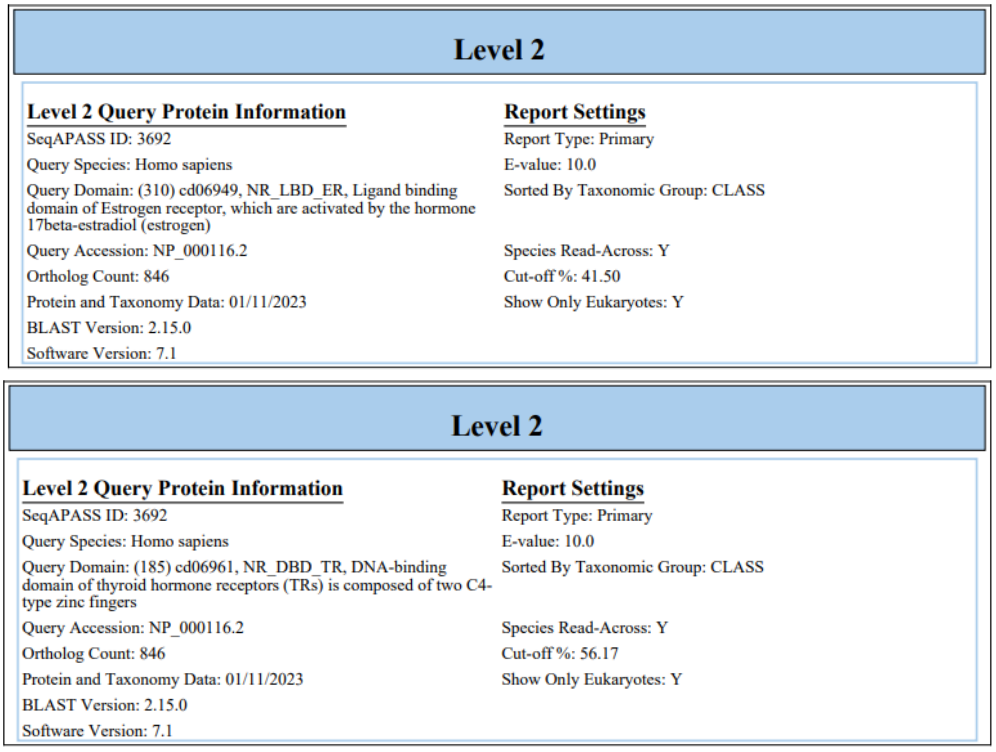
Report Type: Primary
E-value: 0.01
Sorted By Taxonomic Group: CLASS
Common Domains: 1
Species Read-Across: Y
Cut-off %: 34.43
Show Only Eukaryotes: Y

Level 1 Visualization - Primary Report



Level 2 of DS Report PDF

The Level 2 section of the DS Report PDF will contain all the "Level 2 Query Protein Information" along with the Level 2 "Report Settings" for that respective domain's run. The Level 2 information will not be present if no Level 2 run information or Level 2 visualization is pushed to the DS Report PDF. Each domain can have its own respective visualization that can be added to the DS Report PDF by selecting the "Add Visualization to Report" button in the DS Report page.



Level 2

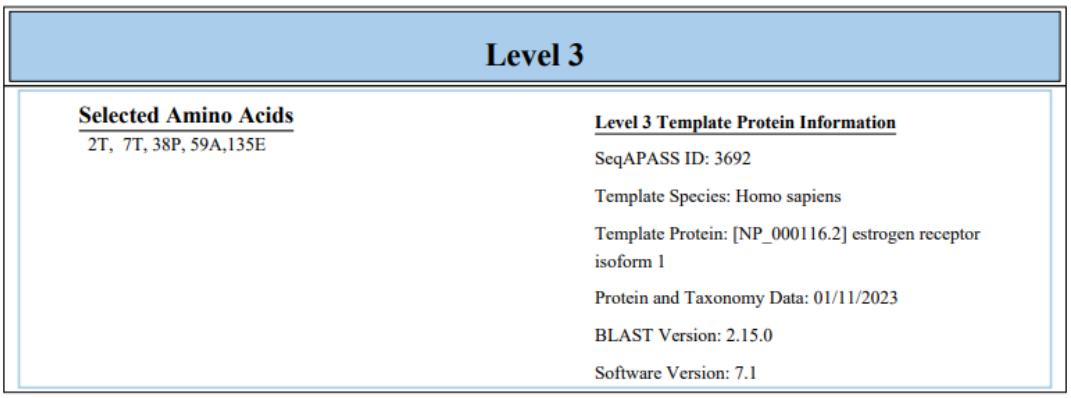
Level 2 Query Protein Information	Report Settings
SeqAPASS ID: 3692	Report Type: Primary
Query Species: Homo sapiens	E-value: 10.0
Query Domain: (310) cd06949, NR_LBD_ER, Ligand binding domain of Estrogen receptor, which are activated by the hormone 17beta-estradiol (estrogen)	Sorted By Taxonomic Group: CLASS
Query Accession: NP_000116.2	Species Read-Across: Y
Ortholog Count: 846	Cut-off %: 41.50
Protein and Taxonomy Data: 01/11/2023	Show Only Eukaryotes: Y
BLAST Version: 2.15.0	
Software Version: 7.1	

Level 2

Level 2 Query Protein Information	Report Settings
SeqAPASS ID: 3692	Report Type: Primary
Query Species: Homo sapiens	E-value: 10.0
Query Domain: (185) cd06961, NR_DBD_TR, DNA-binding domain of thyroid hormone receptors (TRs) is composed of two C4-type zinc fingers	Sorted By Taxonomic Group: CLASS
Query Accession: NP_000116.2	Species Read-Across: Y
Ortholog Count: 846	Cut-off %: 56.17
Protein and Taxonomy Data: 01/11/2023	Show Only Eukaryotes: Y
BLAST Version: 2.15.0	
Software Version: 7.1	

Level 3 of DS Report PDF

The Level 3 section of the DS Report PDF will contain all the "Level 3 Template Protein Information" along with the Level 3 "Selected Amino Acids" for that respective run. This information will not be present if no Level 3 run information or Level 3 visualization is pushed to the DS Report PDF. The run can have a visualization "Heat Map" that can be added to the DS Report PDF by selecting the "Add Visualization to Report" radio button.



Level 3

Selected Amino Acids
2T, 7T, 38P, 59A,135E

Level 3 Template Protein Information

SeqAPASS ID: 3692

Template Species: Homo sapiens

Template Protein: [NP_000116.2] estrogen receptor isoform 1

Protein and Taxonomy Data: 01/11/2023

BLAST Version: 2.15.0

Software Version: 7.1

Final DS Report Table in DS Report PDF

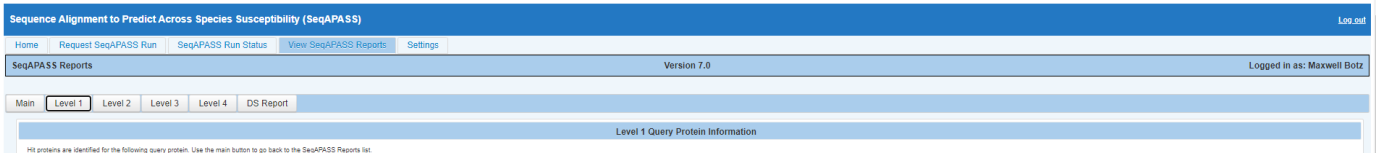
The Final Decision Summary Report table will display the species that were selected for the Level 1 section of the DS report. It can display the specie's respective "Protein", "Level 1 Susceptibility (Y/N)", common domain(s), "Level 3 Template", and "Level 3 Susceptibility" all depending on what is selected from the DS Report set up.

Final Decision Summary Report

Species	Protein	Level 1 Susceptible (Y/N)	(345) cd06157, NR_LBD, The ligand binding domain of nuclear receptors, a family of ligand-activated transcription regulators
Human	estrogen receptor isoform 1	Y	Y
Western gorilla	estrogen receptor alpha	Y	Y
Chimpanzee	estrogen receptor isoform X2	Y	Y
Western lowland gorilla	estrogen receptor isoform X2	Y	Y
Pygmy chimpanzee	estrogen receptor isoform X2	Y	Y
Bornean orangutan	estrogen receptor alpha	Y	Y
Sumatran orangutan	estrogen receptor isoform X2	Y	Y
Sooty mangabey	PREDICTED: estrogen receptor isoform X2	Y	Y
Rhesus monkey	estrogen receptor isoform X2	Y	Y

Moving Between Level 1, Level 2, Level 3, Level 4, and Decision Report Data Pages

As a user chooses to view Level 1, Level 2, Level 3, or Level 4 data in the "View SeqAPASS Reports" tab, new buttons become available for allowing the user to move between Levels of an analysis. The Decision Report data page will become active once a user pushes a finished run using the "Push Level # To DS Report" button.



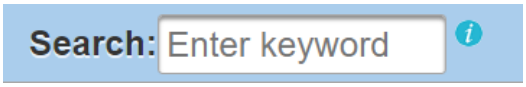
The user can use the "Main" button to return to the list of completed Level 1 runs and select a different query accession to view. The "Level 1" button brings the user to the Level 1 data page, where the user can set up queries for Level 2, Level 3, and if they have access, Level 4, as well as select the button to view Level 2, Level 3, and Level 4 data pages. Open Level 1, Level 2, Level 3, and Level 4 pages remain open until the user selects a different run to view on the "Main" page. Moving between tabs, such as "Home," Request SeqAPASS Run," and "SeqAPASS Run Status", does not close the Level 1, Level 2, Level 3, or Level 4 pages that have been opened.

Note: If the user logs out of the SeqAPASS tool, upon logging back in, the data will reset to default settings. Therefore, the View SeqAPASS Reports tab will not display the "Main," "Level 1," "Level 2," "Level 3," or "Level 4" buttons, until a query is chosen and Level 2, Level 3, and/or Level 4 pages are opened.

Search, View, and Download Data Tables

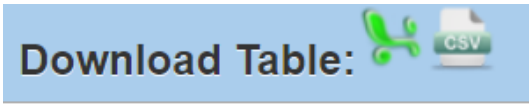
The user can use the "Search" box to enter text to search the table. Further, the user can use the arrow buttons and page numbers on the bottom of the screen to view all data and the drop-down to expand the table to 10, 20, or 50 rows. There are also left and right scroll bars at the bottom of the tables to allow the user to view all columns of the table.

Search using text box on top of tables:

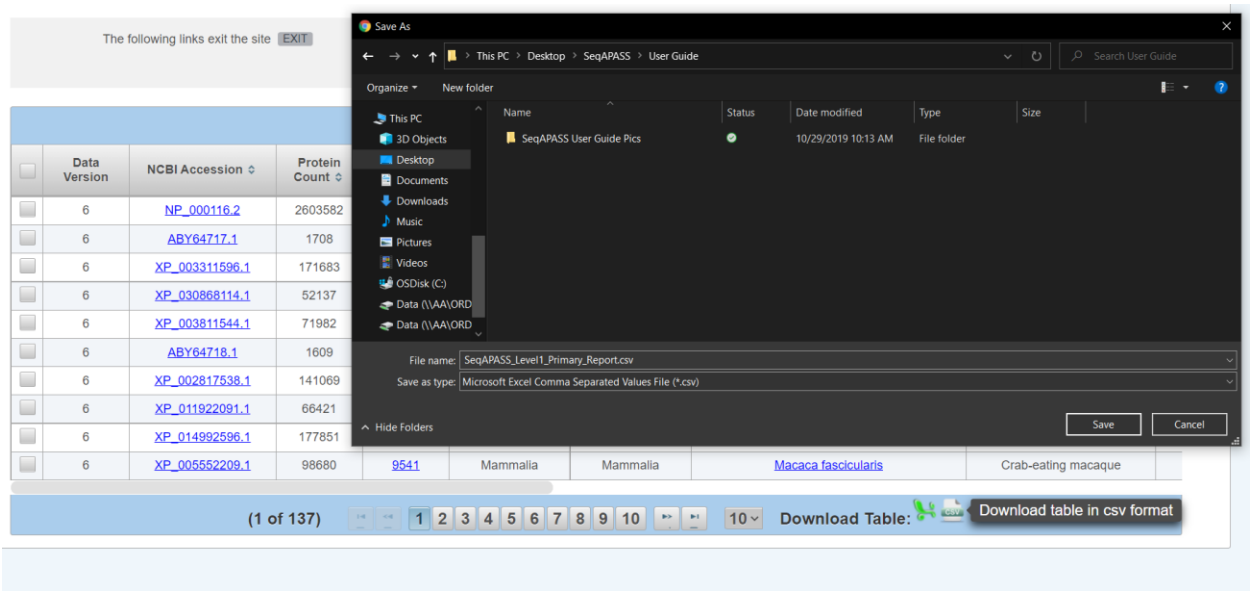


Options for viewing data:

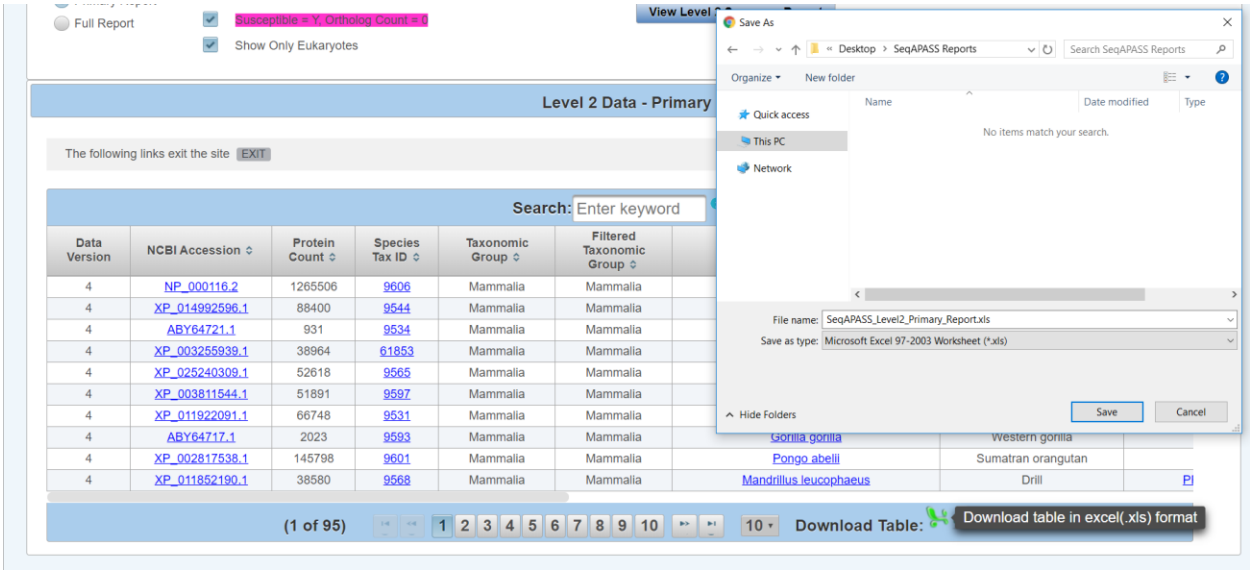
All data tables in the SeqAPASS tool can be downloaded as Excel or csv files. The icons for downloading the files are present on the bottom right-hand side of all tables. Click the icon to download data.



Upon selecting a csv file, the user can choose to save or open the file. Each file is appropriately named by Level of the SeqAPASS evaluation and report type.

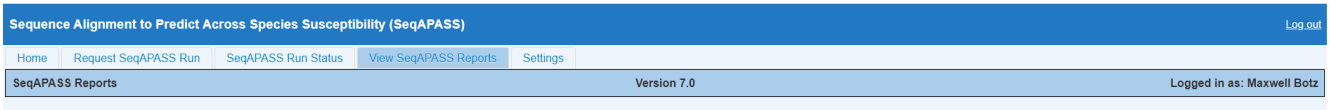


Upon selecting a .xls file, the user can save the report to their desired location. Each file is appropriately named by Level of the SeqAPASS evaluation and report type.



Log out

The user can log out from any page in SeqAPASS, by clicking the "Log out" link on the upper right-hand side of the page. If a user clicks Log out and then Logs back in, all settings will be set back to default. User can log out at any time by clicking the "Log out" link on the upper right-hand side. Any successfully submitted queries that were requested prior to logging out will continue running and when completed, will be available to the user in the "View SeqAPASS Reports" tab.



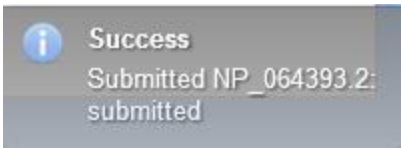
Pop-up Messages

The Spinning Wheel pop-up is used as an indicator to alert the user that an action is taking place, where the interface of the SeqAPASS tool is contacting the backend database. For example, upon clicking the "SeqAPASS Run Status" tab, "Refresh Data" button, "View Level 2 Data" button, or "View Level 3 Data" button the Spinning Wheel will pop-up and disappear from the screen. There are multiple other instances where the spinning wheel is used as an indicator to the user that an action is occurring.

Pop-up messages are meant to guide the user to submit the correct information for a query, inform the user of a successful or failed query submission, or otherwise inform the user of an error. All pop-up messages will appear for 10 seconds on the upper right-hand side of the screen, and then disappear. If the

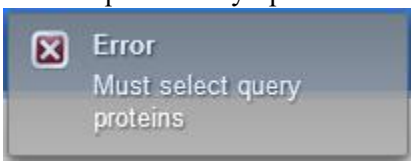
user would like to close the message before the 10 seconds is up, click on the message and an "x" will appear of the upper right-hand corner of the message box. Click the x to close the message.

In the "Request SeqAPASS Run" tab, Compare Primary Amino Acid Sequences "By Species" page, a successful Level 1 query submission will display a pop-up message indicating that the query has been submitted to the run queue or if "existing' message appears indicating that the accession has been ran previously either by a user and is available to view.

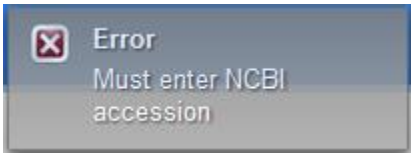


OR

User did not select any query proteins from the "Request SeqAPASS Run" tab, Compare Primary Amino Acid Sequences "By Species" or "By Accession" page, and clicked "Request Run" button.

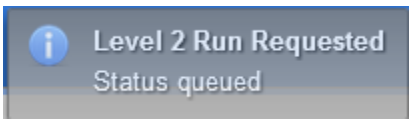


OR



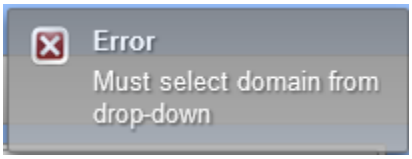
If the user enters non-sense text (or any text that is not an NCBI accession) into the "NCBI Protein Accession" text box for submitting a Level 1 query in the "Request SeqAPASS Run" tab, in the Compare Primary Amino Acid Sequences "By Accession" page, and clicked "Request Run" button, the message below will pop-up indicating that the Accession entered is not in the SeqAPASS database.

In the "View SeqAPASS Reports" tab, Level 1 page, if a user clicks "View Level 2 Data," a successful Level 2 query submission will display a pop-up message indicating that the query has entered the run queue.

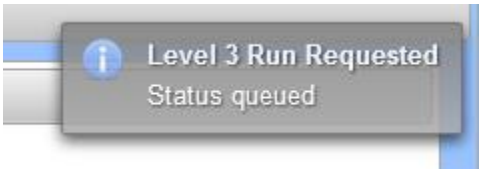


In the "View SeqAPASS Reports" tab, Level 1 page, if a user selects a domain that has already been submitted (but not completed) and clicks "Request Domain Run" a message for successful Level 2 query submission will display a pop-up message indicating that the query has entered the run queue

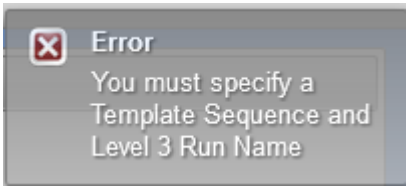
In the "View SeqAPASS Reports" tab, Level 1 page, if a user clicks "View Level 2 Data" without selecting a domain to view from the drop-down, the message below will pop-up to indicate that the user must select a domain.



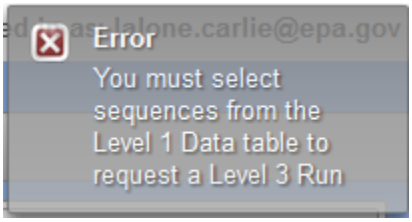
In the "View SeqAPASS Reports" tab, Level 1 page, a successful Level 3 query submission will display a pop-up message indicating that the query has entered the run queue.



In the "View SeqAPASS Reports" tab, Level 1 page, if a user fails to type a user defined Level 3 Run Name, the message below will pop-up to indicate that the user must do so.



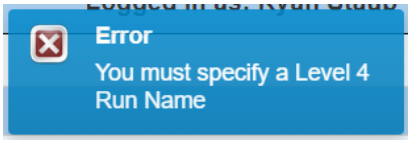
In the "View SeqAPASS Reports" tab, Level 1 page, if a user fails to select species from the Level 1 Data table to be compared with the template sequence, the message below will pop-up.



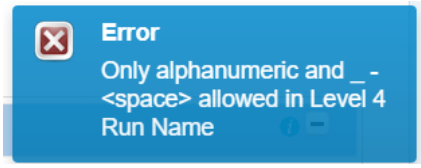
In the "View SeqAPASS Reports" tab, Level 1 page, if a user fails to select a Level 3 Run Name from the Choose Query to View drop-down and clicks the "View Level 3 Date" button, the message below will pop-up.

In the "View SeqAPASS Reports" tab, "Level 3 Template Protein Information" data page, if a user fails to select amino acid residues using the "Select Amino Acid Residues" shuttle and clicks the "View Level 3 Date" button, the message below will pop-up.

In the "View SeqAPASS Reports" tab, Level 1 page, if a user fails to specify a Level 4 Run Name when generating FASTAs for a Level 4 run, a message will pop-up instructing the user to do so.

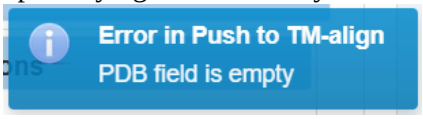


In the "View SeqAPASS Reports" tab, Level 1 page, if a user inputs disallowed characters into the Level 4 Run Name when generating FASTAs, a pop-up will appear instructing the user to change the name to acceptable characters.



In the "View SeqAPASS Reports" tab, Level 1 page, if a user enters a string of characters that do not match the standard PDB:ID format into the "PDBID Restraint" field, a message will pop-up notifying the user of the error.

In the "View SeqAPASS Reports" tab, Level 4 page, if a user fails to appropriately fill in all fields of the "Information for TM-align" section when adding external structures for comparison, a message will pop-up notifying the user of any unfilled fields.

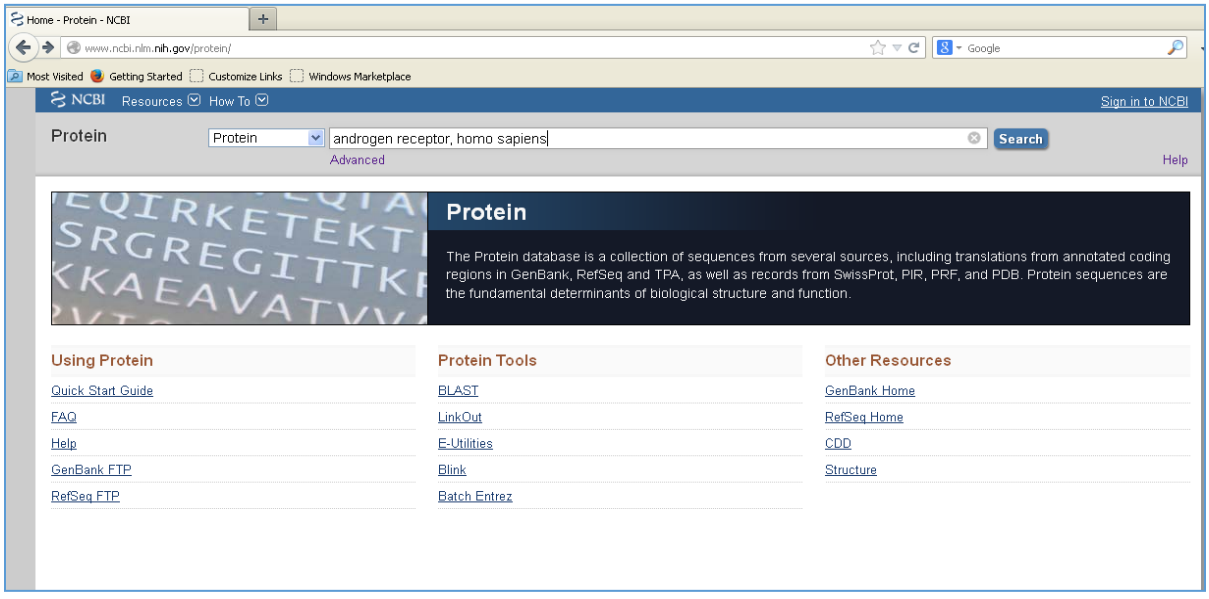


Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS): User Guide
Updated 09/10/2024; Contact Carlie LaLone with Questions: LaLone.Carlie@epa.gov

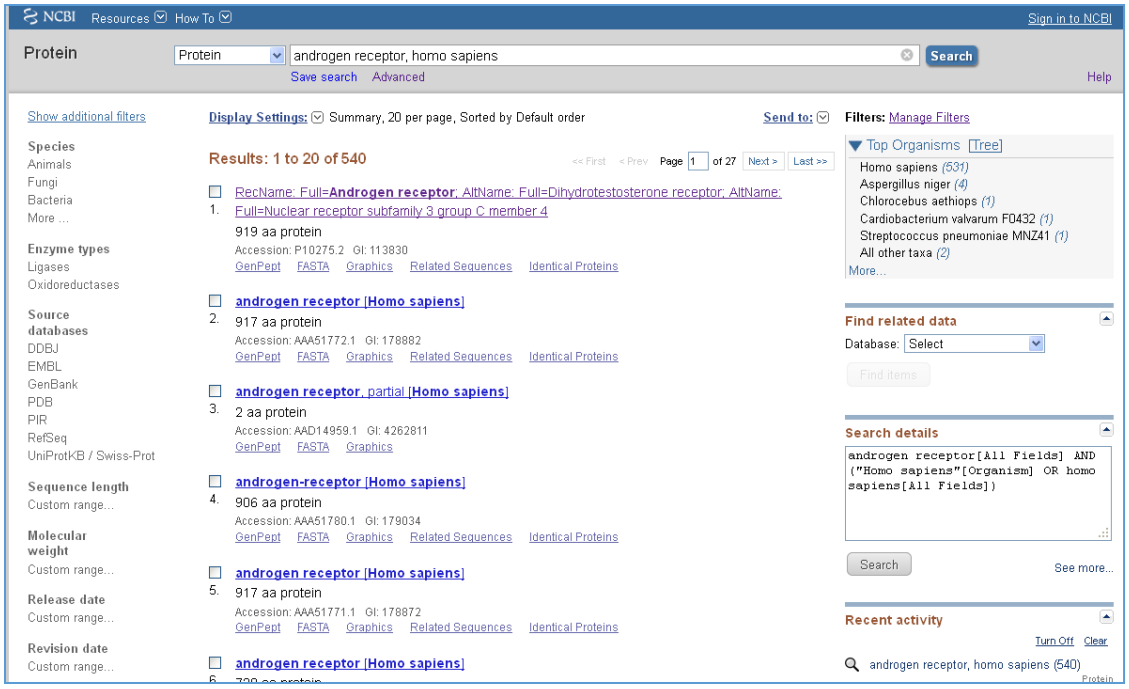
Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) Documentation
Query Species: The selection of the query species for a SeqAPASS analysis is dependent upon the question the user is addressing. For example, the query species can be the target species (i.e., human or companion animal in the case of drugs; or insect, plant, fungus, or pest in the case of pesticides) or, depending on the application of the susceptibility prediction, the query species may be a species known or hypothesized to be sensitive to a chemical acting on the protein molecular target of interest. There may be instances where a protein for the species of interest has not been sequenced, in this case it may serve the users purpose to identify another taxonomically related species from the same organism Class, Order, Family, or Genus as a surrogate query species. In certain cases, when there is interest in the susceptibility of a particular species (e.g., honey bee) and in the case that there are numerous potential target species (e.g., neonicotinoids are intended to cause mortality in a number of pest insects) the species of particular concern may serve as the query species.

Query Protein: SeqAPASS can be queried with any protein sequence available in the NCBI protein GenBank database, by protein name, or NCBI Accession. It is suggested that the user of SeqAPASS examines their query protein and species in the NCBI protein database prior to submitting a run to SeqAPASS (use NCBI link on query page). It is not uncommon for a protein of a specific species to be represented by more than one sequence. In such cases there are some guiding principles for identification of the best sequence available for the SeqAPASS run.

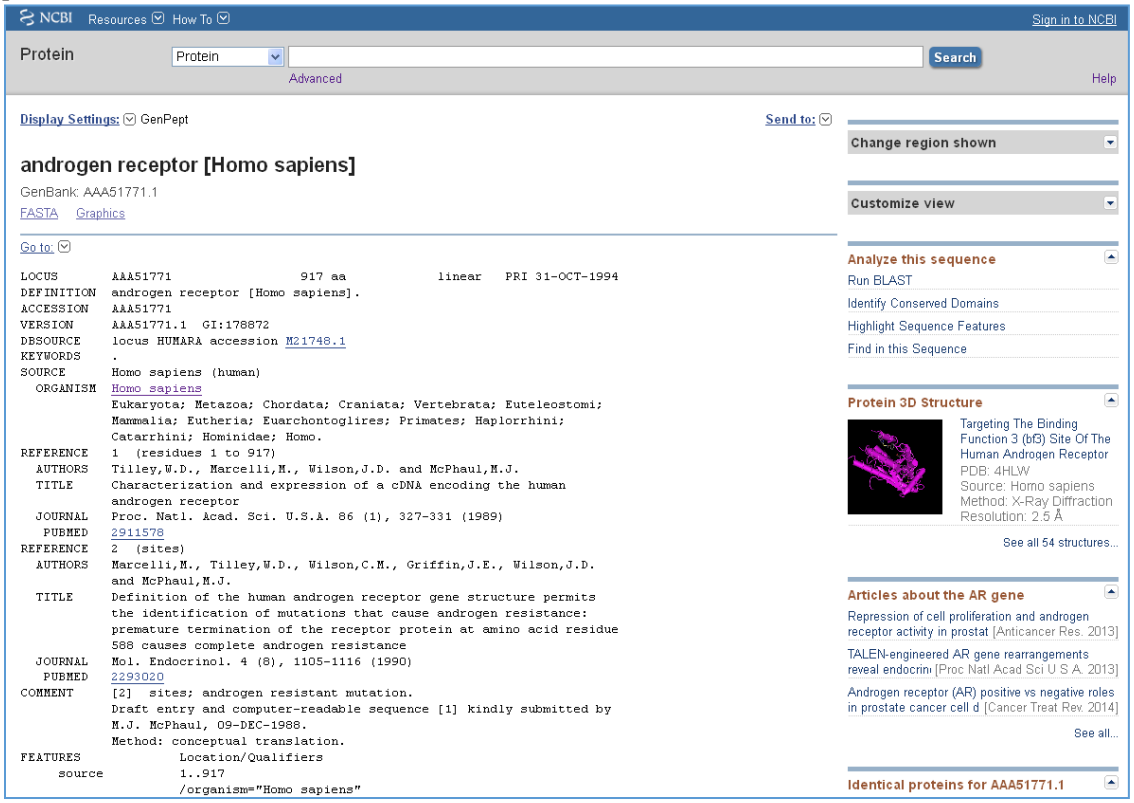
General guidelines: These guidelines describe best practices for identifying the most useful sequence for a species susceptibility prediction in SeqAPASS, however, in some cases, limited sequence information is available and therefore less desirable sequences may be used. It is up to the user of SeqAPASS to recognize the quality and limitations of the sequence chosen for the SeqAPASS query. The information about a particular protein can be found on the Protein page in the NCBI database (http://www.ncbi.nlm.nih.gov/protein/).
http://www.ncbi.nlm.nih.gov/protein/



Search for a protein of interest using protein name and/or species of interest: For the example above, multiple hit proteins were identified.



Select one of the proteins by clicking on the link shown above to see detailed information about the protein

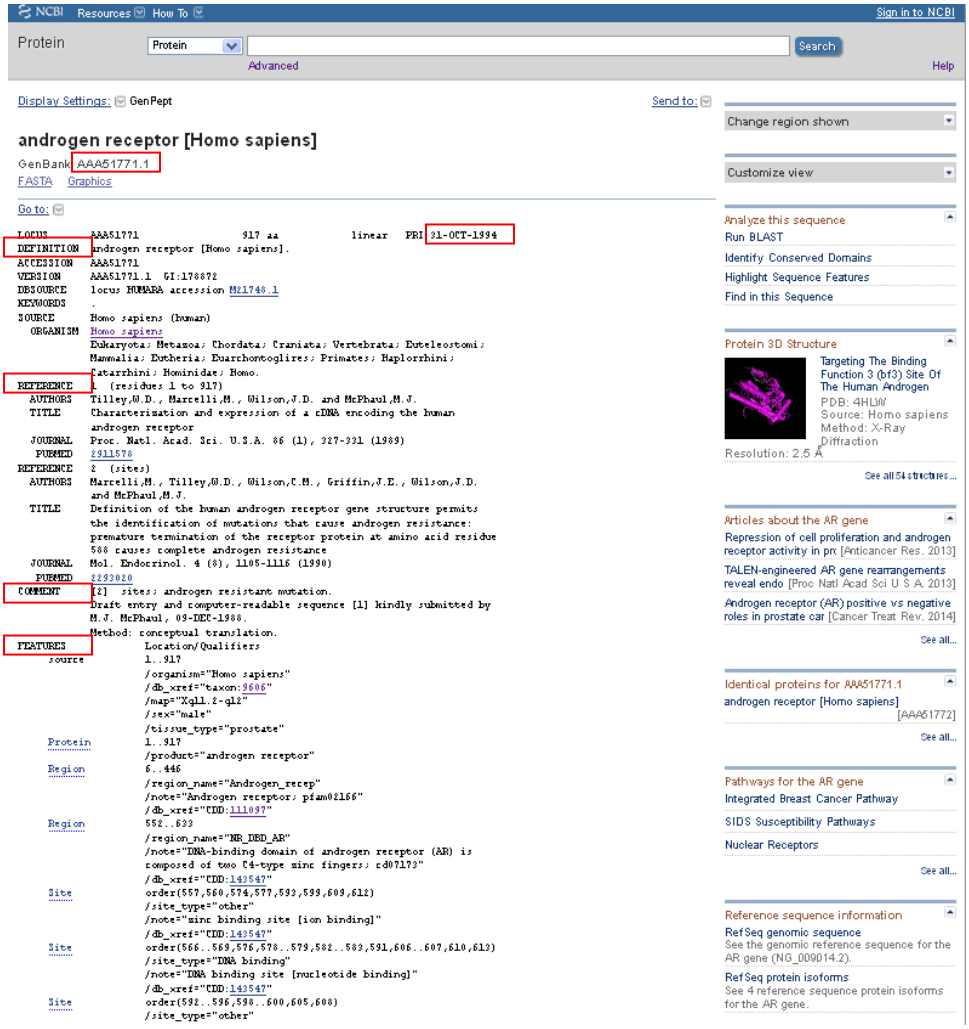


Guiding principles: On the NCBI protein page, rows to examine include: "DEFINITION," "REFERENCES," COMMENTS," and "FEATURES." The information provided in these rows can aid a SeqAPASS user in the identification of an ideal query sequence for SeqAPASS.

It is desirable to:
a. Use accessions with the following prefix: NP_
b. Avoid use of protein sequences labeled "partial," "PREDICTED," "PROVISIONAL," "INFERRED," or "hypothetical"
c. Avoid using those labeled "TPA" (Third Party Annotation), however if TPA is all that is available "TPA: experimental" would be preferred over "TPA: inferential"
d. Look at the date associated with the protein in the "LOCUS" row of the detailed protein page. A more recent date can have the most up-to-date annotation of the protein. Under the "DBSOURCE" row of the detailed protein page other accessions associated with past protein sequences can be viewed. Many times, if the "xrefs" row is heavily populated and has the most recent annotation update date, it is likely to be the best sequence to use as a query sequence in SeqAPASS.
e. Short sequences should be avoided when possible as query sequences. Many times, if one selects the protein from the protein output derived from the NCBI protein database query, they will find that the short sequence is actually a partial sequence described in the "DEFINITION" row of the Protein page.
f. Unless there is reason for doing so (based on the question the user is trying to address), splice-variants labeled in "FEATURES" rows of the Protein page as "alternatively spliced" would be less desirable
g. It is important to check the references associated with the selected query protein. In some cases, certain sequences are associated with sensitivity to a given chemical. This can be particularly useful when predicting susceptibility to pesticides, where certain strains of insects are produced to be readily sensitive or insensitive to a chemical.
h. A secondary check of the sequence used in the SeqAPASS run would be to look at the output derived and see whether ortholog candidates were detected. Ideally a preferential sequence would have more ortholog candidates identified.

Important Note: To identify which query protein has the greatest number of Ortholog Candidates the user can choose to submit multiple proteins with the same species and protein. Upon the Level 1 runs completing for those similar proteins, the user can then select the "View SeqAPASS Reports" tab and look at the table for "Ortholog Count" the protein with the highest number is likely to be the most appropriate query species for a SeqAPASS evaluation.

Example: Androgen receptor, *Homo sapiens*



NCBI Resources How To Sign in to NCBI

Protein Protein Search

Advanced Help

Display Settings: GenPept Send to:

androgen receptor [Homo sapiens]

GenBank AAA51771.1

FASTA Graphics

Go to:

```
LOCUS       AAA51771                917 aa            linear   PRI 31-OCT-1994
DEFINITION  androgen receptor [Homo sapiens].
ACCESSION   AAA51771
VERSION     AAA51771.1  GI:178872
DBSOURCE    locus HUMARA accession M21748.1
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 917)
  AUTHORS   Tilley,W.D., Marcelli,M., Wilson,J.D. and McPhaul,M.J.
  TITLE     Characterization and expression of a cDNA encoding the human
            androgen receptor
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 86 (1), 327-331 (1989)
   PUBMED   2911578
REFERENCE   2  (sites)
  AUTHORS   Marcelli,M., Tilley,W.D., Wilson,C.M., Griffin,J.E., Wilson,J.D.
            and McPhaul,M.J.
  TITLE     Definition of the human androgen receptor gene structure permits
            the identification of mutations that cause androgen resistance:
            premature termination of the receptor protein at amino acid residue
            588 causes complete androgen resistance
  JOURNAL   Mol. Endocrinol. 4 (8), 1105-1116 (1990)
   PUBMED   2293020
COMMENT     [2]  sites; androgen resistant mutation.
            Draft entry and computer-readable sequence [1] kindly submitted by
            M.J. McPhaul, 09-DEC-1988.
            Method: conceptual translation.
FEATURES             Location/Qualifiers
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                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /map="Xq11.2-q12"
                     /sex="male"
                     /tissue_type="prostate"
     Protein         1..917
                     /product="androgen receptor"
     Region          6..446
                     /region_name="Androgen_recep"
                     /note="Androgen receptor; pfam02166"
                     /db_xref="CDD:111097"
     Region          552..633
                     /region_name="NR_DBD_AR"
                     /note="DNA-binding domain of androgen receptor (AR) is
                     composed of two C4-type zinc fingers; cd07173"
                     /db_xref="CDD:143547"
     Site            order(557,560,574,577,593,599,603,612)
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                     /note="zinc binding site [ion binding]"
                     /db_xref="CDD:143547"
     Site            order(566..569,576,578..579,582..583,591,606..607,610,613)
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                     /note="DNA binding site [nucleotide binding]"
                     /db_xref="CDD:143547"
     Site            order(592..596,598..600,605,608)
                     /site_type="other"
```

Change region shown

Customize view

Analyze this sequence
Run BLAST
Identify Conserved Domains
Highlight Sequence Features
Find in this Sequence

Protein 3D Structure
Targeting The Binding Function 3 (bf3) Site Of The Human Androgen
PDB: 4HLW
Source: Homo sapiens
Method: X-Ray Diffraction
Resolution: 2.5 Å
See all 54 structures...

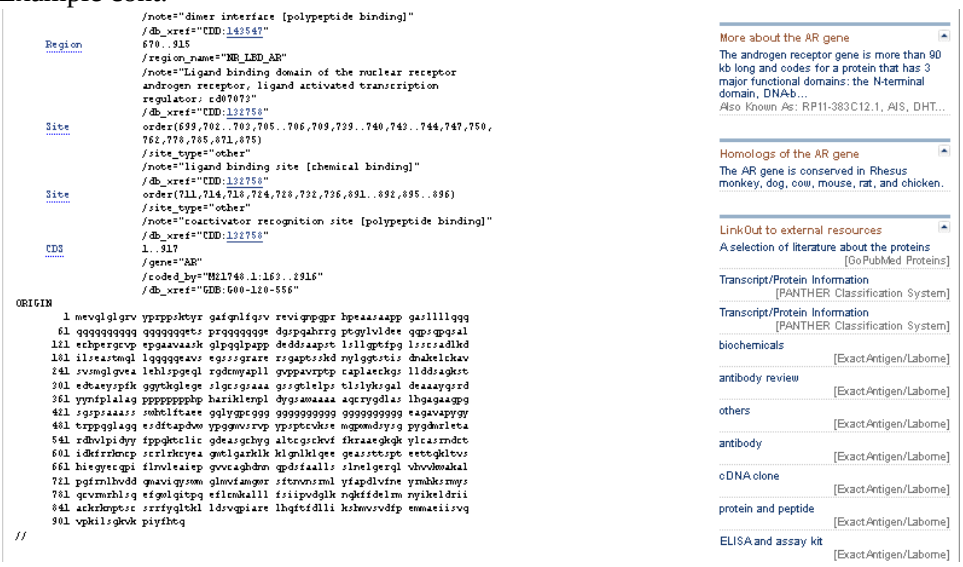
Articles about the AR gene
Repression of cell proliferation and androgen receptor activity in prx [Anticancer Res. 2013]
TALEN-engineered AR gene rearrangements reveal endo [Proc Natl Acad Sci U S A. 2013]
Androgen receptor (AR) positive vs negative roles in prostate car [Cancer Treat Rev. 2014]
See all...

Identical proteins for AAA51771.1
androgen receptor [Homo sapiens] [AAA51772]
See all...

Pathways for the AR gene
Integrated Breast Cancer Pathway
SIDS Susceptibility Pathways
Nuclear Receptors
See all...

Reference sequence information
RefSeq genomic sequence
See the genomic reference sequence for the AR gene (NG_009014.2).
RefSeq protein isoforms
See 4 reference sequence protein isoforms for the AR gene.

Example cont:



```
                     /note="dimer interface [polypeptide binding]"
                     /db_xref="CDD:142547"
     Region          670..915
                     /region_name="NR_LBD_AR"
                     /note="Ligand binding domain of the nuclear receptor
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                     regulator; cd07072"
                     /db_xref="CDD:132758"
     Site            order(699,702..703,705..706,709,729..740,742..744,747,750,
                     762,778,785,871,875)
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                     /note="ligand binding site [chemical binding]"
                     /db_xref="CDD:132758"
     Site            order(711,714,718,724,728,732,736,891..892,895..896)
                     /site_type="other"
                     /note="coactivator recognition site [polypeptide binding]"
                     /db_xref="CDD:132758"
     CDS             1..917
                     /gene="AR"
                     /coded_by="M21748.1:163..2916"
                     /db_xref="GDB:G00-120-556"
ORIGIN
        1 mevqlglgrv yprppsktyr gafqnlfqsv revignpgpr hpeaasapp gaslllqqg
       61 qqqqqqqqqq qqqqqqgets prqqqqqqge dgspqahrrg ptgylvldee qqpsqpqsal
      121 ecpergcvp epgaavaask glpqqlpapp deddsaapst lsllgptfpg lssesadlkd
      181 ilseastmql lqqqqqeavs egssstgrare rsgaptsskd nylggtstis dnakelckav
      241 svsmglgvea lehlspgeql rgdcmyapll gvppavrptp caplaeckgs lldesagkst
      301 edtaeyspfk ggytkglege slgcsgsaaa gsstgtlelps tlslyksgal deaaayqsrd
      361 yynfplalag pppppppphp hariklenpl dygsawaaaa aqcrygdlas lhgagaagpg
      421 sgspsaaass swhtlftaee gqlygpcggg gggggggggg gggggggggg eagavapygy
      481 trppqglagq esdftapdvw ypggmvsrvp ypsptcvkse mgpwmdsysg pygdmrleta
      541 rdhvlpidyy fppgktclic gdeasgchyg altcgskvf fkraaegkqk ylcasrndct
      601 idkfrrknrp scrlrkvyea gwtlgarklk klgnlklqee geasttctpt eettgkltvs
      661 hiegyecqpi flnvleaiep gvvcaghdnn qpdsfaalls slnelgerql vhvvkwakal
      721 pgfrnlhvdd qmavigyswm glmvfamgwr sftnvnvsml yfapdlvfne ymhksrmys
      781 qcvrmrhlsq efgwlqitpq eflcmkalll fsiipvdglk nqkffdelrm nyikeldrii
      841 acknnpttsc srrfyqltkl ldsvqpiare lhqftfdlli kshmvsvdfp emmaeiisvq
      901 vpkilsgkvk piyfhtq
//
```


More about the AR gene

The androgen receptor gene is more than 90 kb long and codes for a protein that has 3 major functional domains: the N-terminal domain, DNA-b...
Also Known As: RP11-383C12.1, AIS, DHT...

Homologs of the AR gene

The AR gene is conserved in Rhesus monkey, dog, cow, mouse, rat, and chicken.

LinkOut to external resources

A selection of literature about the proteins
[GoPubMed Proteins]

Transcript/Protein Information
[PANTHER Classification System]

Transcript/Protein Information
[PANTHER Classification System]

biochemicals
[ExactAntigen/Labome]

antibody review
[ExactAntigen/Labome]

others
[ExactAntigen/Labome]

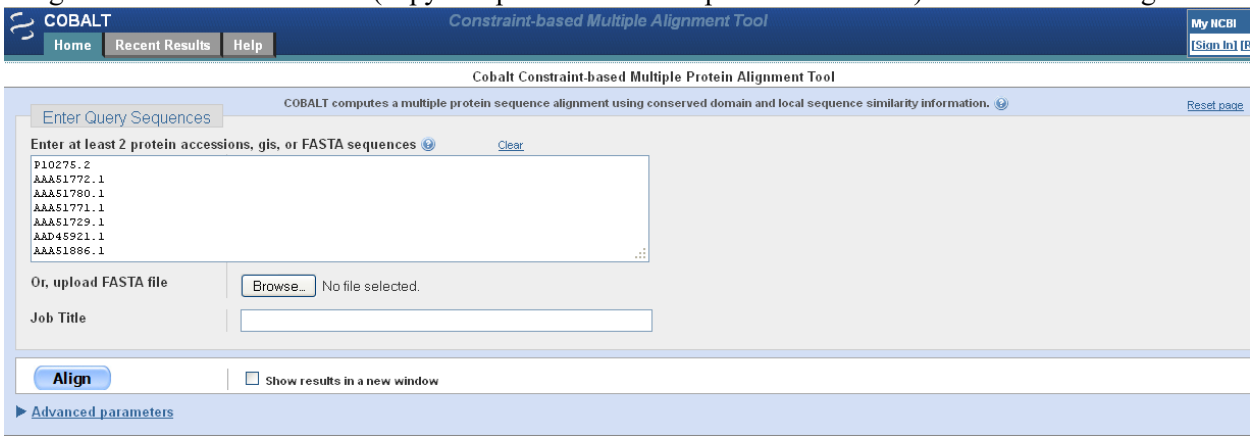
antibody
[ExactAntigen/Labome]

cDNA clone
[ExactAntigen/Labome]

protein and peptide
[ExactAntigen/Labome]

ELISA and assay kit
[ExactAntigen/Labome]

h. If multiple proteins appear to be the best query protein for SeqAPASS, the sequences can be aligned using NCBI's COBALT. Enter (copy and paste from NCBI protein search list) accessions and align.



COBALT — Constraint-based Multiple Alignment Tool

Home | Recent Results | Help

My NCBI [Sign In] [R

Cobalt Constraint-based Multiple Protein Alignment Tool

Enter Query Sequences

COBALT computes a multiple protein sequence alignment using conserved domain and local sequence similarity information.

Reset page

Enter at least 2 protein accessions, gis, or FASTA sequences — Clear

P10275.2
AAA51772.1
AAA51780.1
AAA51771.1
AAA51729.1
AAD45921.1
AAA51886.1

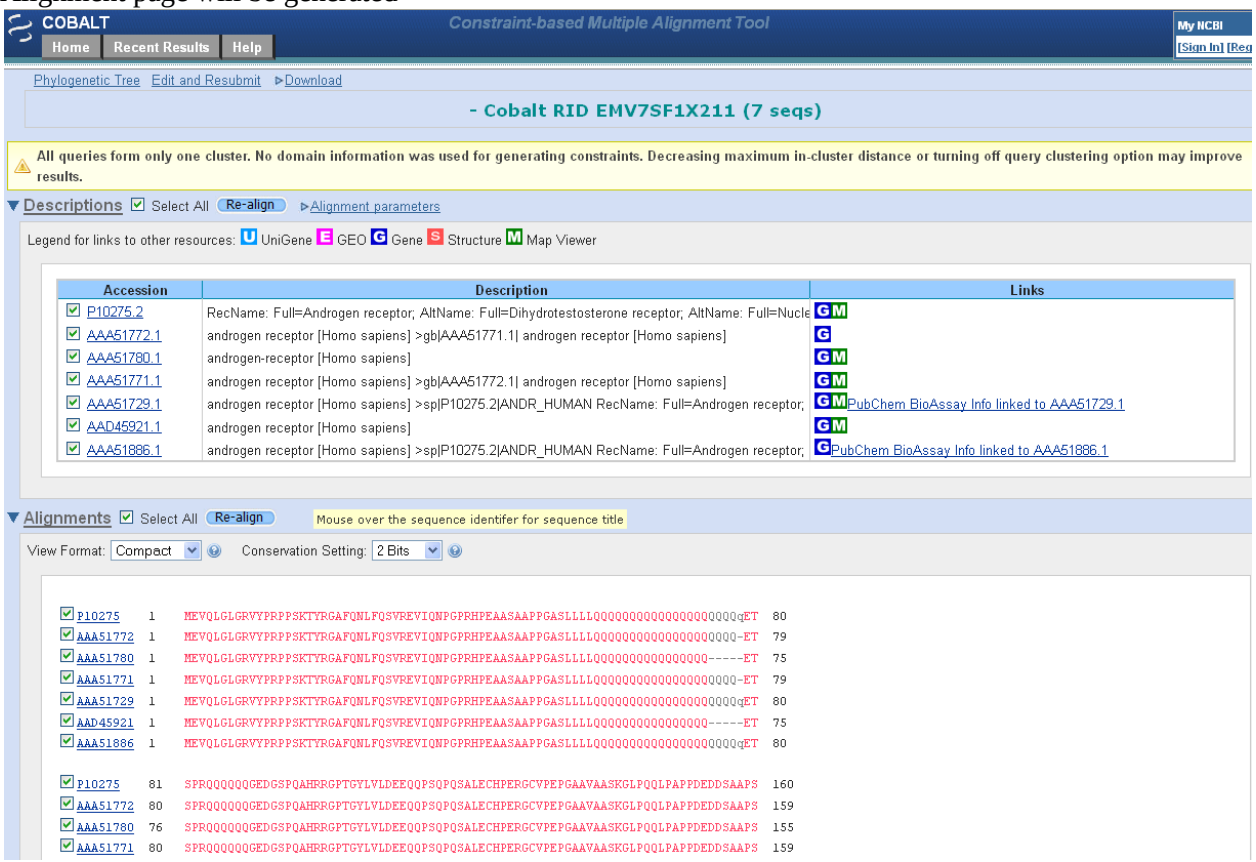
Or, upload FASTA file — Browse... No file selected.

Job Title

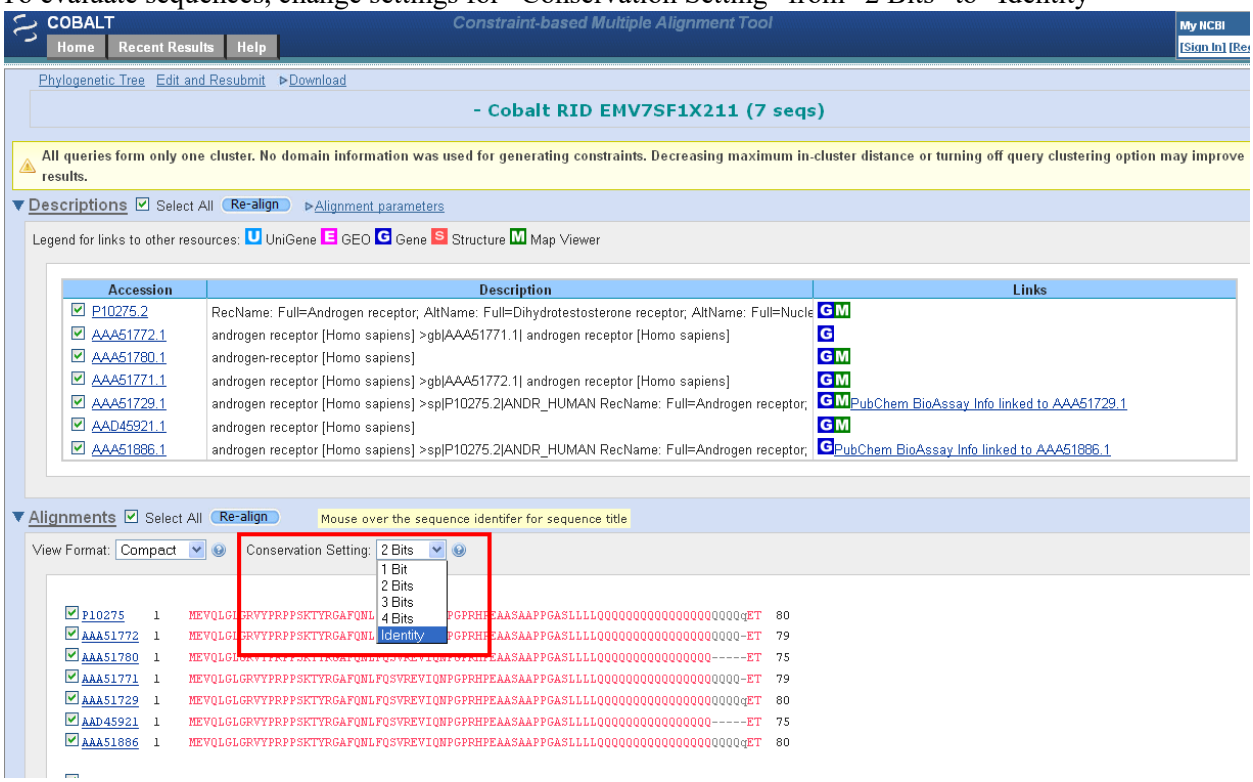
Align — Show results in a new window

Advanced parameters

Alignment page will be generated



To evaluate sequences, change settings for “Conservation Setting” from “2 Bits” to “Identity”



Look for differences in the sequence (e.g., conserved residues, gaps) and start by eliminating sequences that have gaps.
i. If, after the suggested evaluations of the proteins are performed, questions remain as to which sequence would be best to run in SeqAPASS, run all relevant sequences in SeqAPASS for the evaluation. The individual residue differences between commonly named sequences will become most important when evaluating residues known to be important for binding the chemical or activating the protein (Level 3 SeqAPASS analysis). After completing the SeqAPASS run, select the data that has the greatest number of ortholog candidates for your evaluation of conservation and further predictions of cross species susceptibility. Depending on the protein of interest, multiple subunits may be associated with a protein. In this case, all relevant subunits can be queried using SeqAPASS.

Level 1 Calculated Percent Similarity

The SeqAPASS algorithms submit the query to NCBI's standalone BLASTp (using default settings, including BLOSUM-62 matrix), which aligns the query protein with all proteins available in the NCBI protein database and provides a variety of metrics associated with each pairwise alignment between the query and hit sequences. SeqAPASS selectively captures output from BLASTp, including one sequence per species with the highest bit score. Detailed descriptions of metrics derived from BLASTp (e.g., BLASTp Bitscore, E-Value, Positives, Identity, Hit length) can be found in:
The NCBI Handbook: (http://www.ncbi.nlm.nih.gov/books/NBK21106/);
BLAST® Help: (http://www.ncbi.nlm.nih.gov/books/NBK62051/) and the
NCBI Glossary Field Guide: (http://www.ncbi.nlm.nih.gov/Class/FieldGuide/glossary.html)

The top row of the Level 1 data corresponds to the queried protein selected by the user. For each sequence queried, the Level 1, top row query sequence is used to determine the maximum bitscore for the analysis, which is derived from aligning the query sequence to itself using BLASTp. To calculate percent similarity, the bitscore for each hit sequence is normalized to the maximum bit score and then multiplied by 100.

Note: SeqAPASS v2.0 and newer parse the BLASTp query and hit accessions to identify all the species/accessions from the identical proteins. Therefore, if a hit sequence represents multiple species, all species with the identical sequence will be found in the data table for Level 1 and Level 2. To determine which sequence/species was identified from BLASTp as a hit and which sequence/species was parsed from the identical sequence, view the "Full Report" for Level 1 or Level 2, column "Identical Protein," Where "N" is indicative of the original hit sequence and "Y" is the parsed sequence.

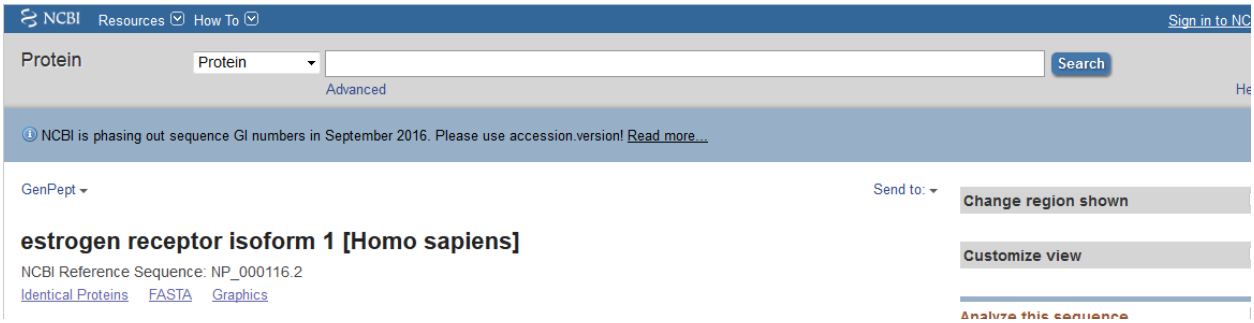
Common Domain Count

Reversed Position Specific BLAST (RPS BLAST) is used to compare each query and hit sequence to conserved domains defined in NCBIs Conserved Domain Database. A hit domain is considered in common with the query domain if it contains the same domain accession as the query and it aligns with the NCBI curated domain with the same or greater amino acid residue coverage than the query sequence.

Ortholog Candidate Identification

Ortholog sequences are those that have diverged from a speciation event and therefore are more likely to maintain similar function. SeqAPASS uses reciprocal best hit (RBH) BLAST for ortholog detection by automatically comparing each hit protein to all protein sequences available for the query species and if the original query protein or one of its identical protein matches is identified to by the best match to the hit or maintain the same bitscore, then the hit sequence would be considered an ortholog candidate. The sequence is indicated an Ortholog Candidate or not with a yes (Y) or no (N) in the column.

Note: Many NCBI protein accessions represent multiple identical protein sequences in the BLASTp output. This is due to BLASTp querying and presenting data from the non-redundant protein database. Sometimes the identical sequences are from different species. This can be checked by following the link for the top row "NCBI Accession" in the table to the NCBI protein page. Below the protein name [species] title will be a link to "Identical Proteins."
Click the "Identical Proteins" link and look for a sequence in the list from the user defined query species.



Note: If the top hit is a Protein DataBank (PDB) code (e.g., 1AHR_A) from RBH BLAST there will be no ortholog candidates identified. BLASTp when ran against all accessions for a given species does not return PDB codes. It is recommended that the user identify a similar/identical sequence to the PDB code and use that sequence as the query sequence.

Susceptibility cut-off
The susceptibility cut-off values listed on the "Level 1 (and Level 2) Susceptibility Cut-off" page are determined by plotting the % similarity data from the "Primary Report" or "Full Report" and identifying the local minimums in the data. The default cut-off is determined by taking the 1st local minimum and moving up in percent similarity until the next ortholog candidate is found. The susceptibility cut-off displayed in the list is the percent similarity of the identified ortholog candidate.

Criteria for Susceptibility Prediction (when "Primary Report Settings" is set to "Species Read-Across:" Yes)

All sequences identified above the susceptibility cut-off are predicted to be susceptible; therefore, Susceptibility Prediction = Y for "yes"

If the hit sequence is below the susceptibility cut-off, but identified as an Ortholog Candidate = Y, for "yes," then the hit is predicted to be susceptible; therefore, Susceptibility Prediction = Y for "yes"

If the hit sequence is below the susceptibility cut-off but belongs to any organism class found above the susceptibility cut-off, the hit is predicted to be susceptible; therefore, Susceptibility Prediction = Y for "yes". This criterion allows susceptibility predictions to be made across taxonomic groups based on the likelihood that the sequences above the cut-off are better matches to the query.

If the hit sequence is below the susceptibility cut-off and not identified as an ortholog candidate (Ortholog Candidate = N, for "no,") and does not belong to any organism class found above the susceptibility cut-off, the hit is predicted to not be susceptible; therefore, Susceptibility Prediction = N for "no"

Note that the "Primary Report" may yield different Susceptibility Predictions than the "Full Report," as the predictions are based on the data in the different reports. The Primary Report is filtered to only display E-value ≤ 0.01 and Common Domain Count ≥ 1.

Criteria for Susceptibility Prediction (when "Primary Report Settings" is set to "Species Read-Across:" No)

All sequences identified above the susceptibility cut-off are predicted to be susceptible; therefore, Susceptibility Prediction = Y for "yes"

If the hit sequence is below the susceptibility cut-off, but identified as an Ortholog Candidate = Y, for "yes," then the hit is predicted to be susceptible; therefore, Susceptibility Prediction = Y for "yes"

If the hit sequence is below the susceptibility cut-off and not identified as an ortholog candidate (Ortholog Candidate = N, for "no,"), the hit is predicted to not be susceptible; therefore, Susceptibility Prediction = N for "no"

Level 2 Calculated Percent Similarity

Data obtained from the Level 1 RPS BLAST evaluation is used to assign sequence ranges that aligned with a user selected domain (from the NCBI CDD database) to each accession from the Level 1 Full report. BLASTp is then used to align the query domain range to each hit domain range. The percent similarity is calculated based on the bit scores from the BLASTp alignment of the domain regions. For each sequence queried, the Level 2, top row query species is used to determine the maximum bitscore for the analysis, which is derived from aligning the query sequence to itself using BLASTp. To calculate percent similarity, the bitscore for each hit sequence is normalized to the maximum bit score and then multiplied by 100.

Susceptibility cut-off (same method as used in Level 1)

The susceptibility cut-offs listed on the "Level 2 Susceptibility Cut-off" page are determined by plotting the % similarity data from the "Primary Report" or "Full Report" and identifying the local minimums in the data. The default cut-off is determined by taking the 1st local minimum and moving up in percent similarity until the next ortholog candidate is found. The susceptibility cut-off displayed in the list is the percent similarity of the identified ortholog candidate.

Level 2 Criteria for Susceptibility Prediction (when "Primary Report Settings" is set to "Species Read-Across:" Yes)

All sequences identified above the susceptibility cut-off are predicted to be susceptible; therefore, Susceptibility Prediction = Y for "yes"

If the hit sequence is below the susceptibility cut-off, but identified as an Ortholog Candidate = Y, for "yes," then the hit is predicted to be susceptible; therefore, Susceptibility Prediction = Y for "yes"

If the hit sequence is below the susceptibility cut-off but belongs to any organism class found above the susceptibility cut-off, the hit is predicted to be susceptible; therefore, Susceptibility Prediction = Y for "yes". This criterion allows susceptibility predictions to be made across taxonomic groups based on the likelihood that the sequences above the cut-off are better matches to the query.

If the hit sequence is below the susceptibility cut-off and not identified as an ortholog candidate (Ortholog Candidate = N, for "no,") and does not belong to any organism class found above the susceptibility cut-off, the hit is predicted to not be susceptible; therefore, Susceptibility Prediction = N for "no"

Note that the "Primary Report" may yield different Susceptibility Predictions than the "Full Report," as the predictions are based on the data in the different reports. The Primary Report is filtered to only display E-value ≤ 0.01 and Common Domain Count ≥ 1.

Level 2 Criteria for Susceptibility Prediction (when "Primary Report Settings" is set to "Species Read-Across:" No)

All sequences identified above the susceptibility cut-off are predicted to be susceptible; therefore, Susceptibility Prediction = Y for "yes"

If the hit sequence is below the susceptibility cut-off, but identified as an Ortholog Candidate = Y, for "yes," then the hit is predicted to be susceptible; therefore, Susceptibility Prediction = Y for "yes"

If the hit sequence is below the susceptibility cut-off and not identified as an ortholog candidate (Ortholog Candidate = N, for "no,"), the hit is predicted to not be susceptible; therefore, Susceptibility Prediction = N for "no"

Level 3 Sequence Alignments

COBALT is used to align all user selected sequences (from Level 1 hits) with a user defined template sequence. Because COBALT algorithms align all sequences, it is recommended that the user align the template sequence with sequences that are most similar to one another. As a means to capture the most similar sequences from the SeqAPASS data it is recommended that the user filter the Level 1 data by taxonomic group and step through the Level 1 data pages one by one while selecting sequences. It is recommended that the user look at the name of the sequence and exclude 'partial' sequences when possible. Requesting a query from one taxonomic group at a time, breaks the data down in manageable alignments.

Selecting Amino Acid Residues to Align

The user may select up to 50 amino acid residues to compare across selected species in Level 3.

Level 4 Structural Alignments

Level 4 is intended for the use by expert users only. It is recommended that each user only submit ten I-TASSER jobs at a time and wait for them to complete prior to submitting more. Although it is inconvenient the user must begin the Level 4 request process over beginning with submitting a user defined name for the job, generating FASTA, and submitting the next 10 FASTA to I-TASSER. A recommended naming format could be by Protein accession followed by 1_10; Protein accession 11-20, etc. The user should prioritize which jobs they need completed first. The I-TASSER jobs take days to complete, therefore it is important for the expert users of the tool to not overwhelm the system by submitting too many jobs at once. Proteins that have been submitted to I-TASSER previously within a query will be marked as submitted. The user should pay attention to which I-TASSER jobs have been submitted previously.

TM-align

The TM-align susceptibility prediction is “Y”, indicating yes, if the % similarity is >20%. Below 20% the susceptibility call is “N”, indicating no.