



iRepertoire, Inc.

iPair Analyzer™ User's Guide

Cognate, physical single cell pairing of receptor chains from NGS data

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Minor	Updated spelling errors	V20201009



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Introduction

The immune repertoire amplification of T-cell receptors coupled with next-generation sequencing provides detailed, sequence-level insight into the immune system. However, information related to the cognate pairing of alpha and beta chains or Ig heavy and light chains is lost once RNA extraction is performed on the bulk sample. iRepertoire has developed a sensitive method that allows for the amplification of both TCR alpha and beta chains or BCR heavy and light chains from single cells using arm-PCR all in the same reaction well. The purpose of the iPair Analyzer™ is to aid with the visualization of the NGS data generated after this process is complete.

The iPair Product & Service

For those familiar with iRepertoire's arm-PCR technique, the iPair™ method follows a similar two stage process: During PCR1, RT-PCR is performed with nested, multiplex primers covering both loci with communal forward and reverse binding sites included on the 5'-end of the inside primers (Figure 1). Included on the C-region gene primer is an in-line barcode, which serves as a plate identifier so that multiple 96-well plates may be multiplexed on a sequencing flow cell. After RT-PCR1, the products are rescued. PCR2 is performed with dual-indexed primers that complete the adaptors introduced during PCR1 and provide plate positional information (Figure 2). In this step, each well, and thus each single cell, is uniquely barcoded. In some cases, bulk next-generation sequencing of each chain is also performed on RNA from remaining cells (those cells not subjected to single cell plating). Therefore, the iPair Analyzer™ also facilitates easy comparisons to the bulk data. Any gene amplified in a given well can be traced back to that well, thereby allowing the addition of phenotypic genes, which are available in iPair+ panels.

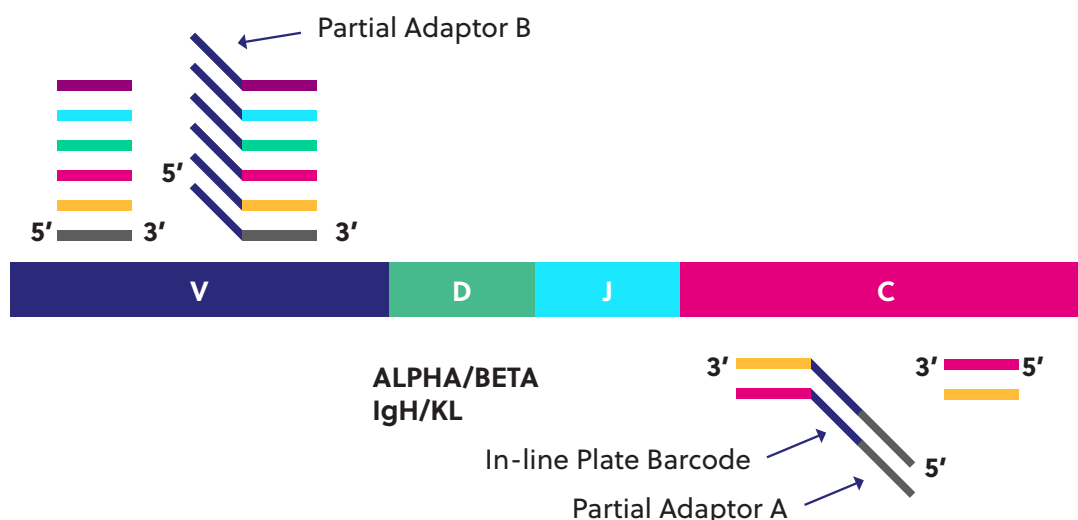


Figure 1. Nested RT-PCR1 is performed with both outside and inside primers covering the VDJ rearrangements of both the TCR alpha and beta locus or Ig heavy and kappa/lambda. The inside reverse primers contain a molecular barcode that serves to identify the 96-well plate. Multiple 96-well plates may be multiplexed, or pooled, onto the same flow cell. A common tag is associated with both the forward and the reverse primers so that these can be used during the second round PCR process.

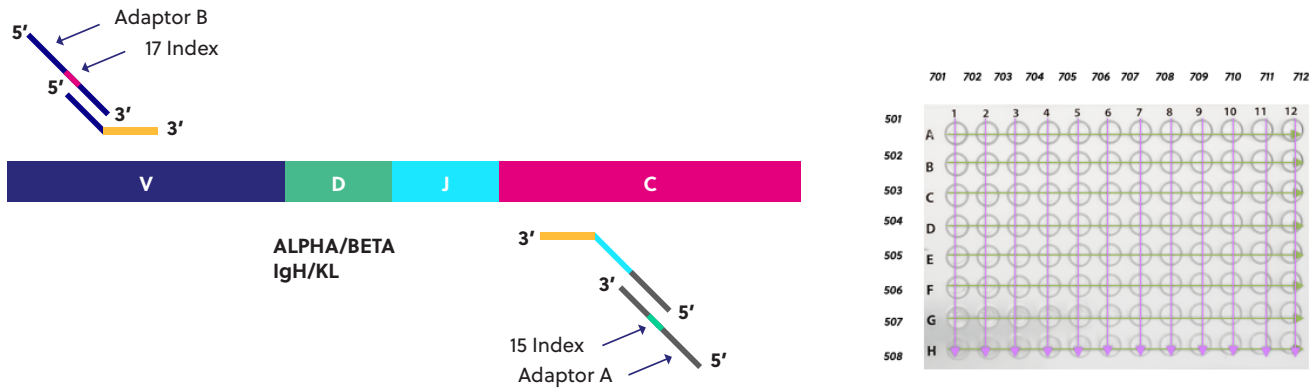
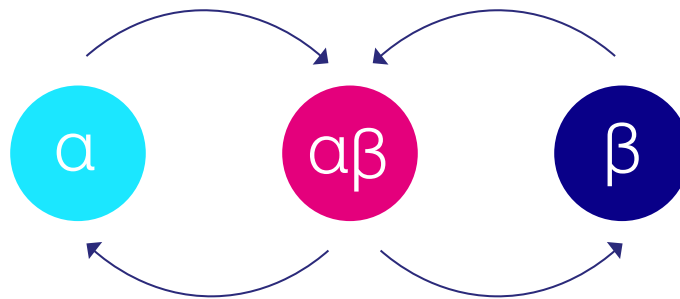


Figure 2. During PCR2, the iDual PCR2 plate is used, and the libraries generated are labelled with forward and reverse index adaptors compatible with the Illumina dual-index chemistry. Libraries generated after PCR2 are sequencing ready and require no additional enzymatic steps in order to complete the sequencing.

iPair Analysis™ Tool

In an effort to be able to derive meaning from the sheer amount of data available from next-generation sequencing, we have made a software that aids in understanding and displaying the data for each cell. During iPair™ processing, sometimes bulk next-generation sequencing is performed alongside the cognate pairing (optional). The bulk data is ranked by dominance based on frequency of reads. Single cell alpha and beta chains are independently compared to the ranked-bulk sequencing data in order to find those highly dominant pairs which may be of significance. The software also helps with parsing the data for downstream use and additional comparisons in spread sheet software.





Software Installation and Demo Access

To access the demo version of the iPair Analyzer™, please contact iRepertoire's Customer Service at info@irepertoire.com or complete the request form available on our website, www.irepertoire.com. Once either a request is sent to Customer Service or a Request Form submitted, credentials will be sent to a valid email address for access to the software, sample data, and a copy of this manual.

Minimum System Requirements

WINDOWS or MAC COMPATIBLE

OS	64-bit: Windows 2000, Windows XP, Windows Vista, Windows 7, Windows 8 Mac OS X 10.10 Yosemite, Mac OS X 10.11 El Capitan
CPU	Intel Celeron 2.16 GHz (2 Core)
Memory	1.88GB useable RAM
Hard Drive	4.48GB free
Resolution	1024 x 768
Color Mode	16-bit/65536

Getting Started

To start, download the iPair ZIP file of the iPair Analyzer™, containing the latest version for your operating system from iRepertoire's website. Extract the ZIP file's contents to reveal an iPairSetup.exe file (for Windows), and a directory containing a sample project. Inside the sample project directory are gzipped CSV files containing the sample data. Feel free to look inside these files, but do not modify or remove the original gzipped CSV files. This sample directory can be read in the iPair, so it is recommended that you save the directory in your preferred working directory.

Windows Installation: Double-click on the **iPairSetup.exe** file to activate the installation wizard, and follow the on screen steps in order to complete the software installation. Once installed, you can find the iPair Analysis Tool shortcut in the Windows Start Menu. To open the iPair Analyzer™, double-click the iPair icon.

Mac Installation: Double click on the **iPair.dmg** file to open a window containing the iPair.app file and the Applications folder. Click and drag the iPair.app icon into the Applications folder to install the iPair Analyzer™, and then move the iPair.dmg file to the trash. Once installed, the iPair Analyzer™ can be opened from the Applications folder.



Overview of the GUI window

The following image briefly summarizes the different portions of the graphical user interface to demonstrate the layout features. There are 5 major panels. The panel labelled "A" below is the toolbox. More details will be provided later in the manual regarding the features that can be overlaid onto the plate through Panel A. The Central Panel (B) shows the project being displayed and a visual output of a 96-well plate. When different analyses are selected in the Toolbox, the overlay will be displayed in the Central Panel. Panel C shows the single cell results of the alpha chain once a square in the 96-well plate of the Central Panel is selected. Panel D shows the single cell results of the beta chain once a square in the 96-well plate of the Central Panel is selected (or heavy and light chains for BCRs). Panel E should be considered like a worksheet. Interesting alpha and beta chains can be loaded into this panel in order to view additional features of the receptor such as the nucleotide sequence of the read, CDR1, CDR2, CDR3, V, D, and J usage. This panel can be exported to CSV for downstream analysis.

A. Toolbox

- Analysis X Export
- Show TRA
- Show TRB
- Gradient
- Overlay uCDR3
- Read Count
- Rank
- Twilight Sky
- TRA
- TRA & TRB
- TRB

B. Central Panel

Open Project Analysis iPair+ Barcode 58

	701	702	703	704	705	706	707	708	709	710	711	712
501	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
502	B01	B02	B03	B04			B07	B08	B09	B10	B11	B12
503	C01	C02	C03	C04	C05	C06	C07	C08	C09		C11	C12
504	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10		D12
505	E01	E02	E03		E05	E06	E07		E09	E10	E11	E12
506	F01	F02	F03	F04		F06	F07	F08	F09	F10	F11	F12
507	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
508		H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

TRA & TRB: 46 Wells: 88

C. Alpha Single Cell

TRA A01 Demo

CDR3 Peptide	Read Count
ALVPNTNTGKLT	6058
ALSDGTGGYKVV	2893
ALAPNTNTGKLT	263

D. Beta Single Cell

TRB A01 Demo

CDR3 Peptide	Read Count
ASGDATVGNQAPL	409

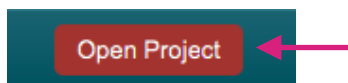
E. Selected Unique CDR3s Worksheet

Well	Sample Name	CDR3 Peptide	V	D	J	C	+
A01	Demo	ASGDATVGNQAPL	mTRBV13-2	*	mTRBJ1-5	mTRBC2	
A01	Demo	ALVPNTNTGKLT	mTRAV6-7/DV9	*	mTRAJ27	mTRAC	



Loading and Viewing a Project

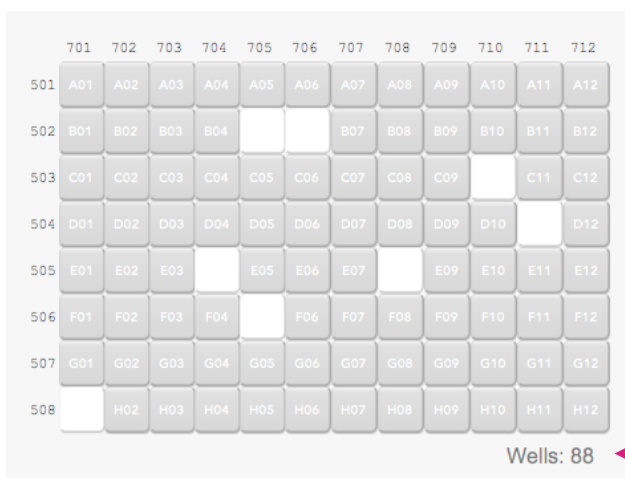
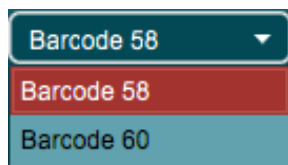
At the start, the iPair Analyzer™ will be empty since no project has been loaded into it. To load a project, select the top button that by default says <Choose Project>, which will activate a directory chooser. Find the location of where you saved the sample project (or any other iPair compatible project) and open the directory. File names for projects should contain the extension .ipair. The project will begin loading in the window, and once finished, the data will be displayed in the iPair Analysis.



By default, the iPair analysis will compare the selected project's data to its own Bulk data (if available). If the project fails to load, please ensure the selected directory is an iPair project. If an iPair project has become unreadable, please see the Contact Information section for more assistance.

Central Panel 96-well Plate

When a project is loaded, the plate should fill up with grey wells or colored wells depending on defaults set. The grey or colored wells are wells that contain unique CDR3s (uCDR3s) within them. Below the plate is the number of wells containing uCDR3s.



number of wells containing uCDR3

If multiple 96-well plates were performed for the same sample, the different plates are accessible from the Plate BC drop-down menu which is found next to the selected project.



Chain-Specific Panel Features

Selecting a well will bring more information about the well in the top two tables on the right, as shown below with a TCR alpha and beta as an example.

Alpha B05 Sample BC03			Beta B05 Sample BC03		
CDR3 Peptide	Read Count	BULK Rank +	CDR3 Peptide	Read Count	BULK Rank +
IVRDNNNDMR	28152	1	ASGQGNYGYT	9456	4

Each table is titled by its respective chain. In this example, this project contains Alpha and Beta chains, so the tables are titled as such. The tables are also titled by the well selected, and in this case, well B04 was selected. By default, the table displays columns for the CDR3 Peptide, the Read Count, and the rankings found in the **Bulk** if bulk data exists. Since multiple samples can be included on the same plate, the sample name for each well is also included here.

The read count refers to the stitched and filtered reads associated with that particular uCDR3 from that particular well (and thus single cell). The bulk sample refers to the remaining cells from a sample that did not undergo single cell analysis. For the bulk sample, the remaining cells RNA were extracted and amplified using iRepertoire’s bulk primer sets with at least one amplification for the alpha chain and one for the beta chain (a minimum of 2 libraries). The unique CDR3 of each chain was ranked based on dominance (frequency of uCDR3). The bulk rank is the position that the single cell CDR3 showed up in or matched with the bulk sample. For instance, in Alpha B05 in the example above the CDR3 peptide “IVRDNNNDMR” matched the top ranked uCDR3 in the remaining bulk cells. However, the Beta uCDR3 “ASGQGNYGYT” from the same well matched the fourth top ranked uCDR3 in the remaining bulk cell. If a particular uCDR3 sequence was not picked up during the bulk sequencing the bulk rank is designated as N/A. More details regarding these features will be discussed during the **Analysis** section of this manual.

If the tables are aligned horizontally, you can adjust the widths of the tables by adjusting the vertical bar in between the tables. If the tables are aligned vertically, then you can adjust the height of the tables by adjusting the horizontal bar found between the tables. At any time, users can click the + in the top right corner of the table in order to show/hide columns.

Alpha B05 Sample BC03			Beta B05 Sample BC03		
CDR3 Peptide	Read Count	BULK Rank +	CDR3 Peptide	Read Count	BULK Rank +
IVRDNNNDMR	28152			9456	4

- CDR3 Peptide
- Read Count
- C
- BULK Rank
- BULK Rank



Selected Unique CDR3 Worksheet

For more information on a particular **uCDR3s**, double-click on the entry in the table. This will fill the selected unique CDR3 table found at the bottom of the iPair Analyzer.

Selected Unique CDR3s						
Well	Sample Name	CDR3 Peptide	V	D	J	C +
B05	Sample BC03	ASGQGNYGYT	hTRBV3-1	hTRBD1	hTRBJ1-2	hTRBC1
B05	Sample BC03	IVRDNNNDMR	hTRAV26-1	*	hTRAJ43	hTRAC

By default, the table displays the Well, CDR3 Peptide, V gene, D gene, J gene, C gene. Once again, click the + in the top right corner to open the table menu and to show/hide columns. The Joined Nucleotide, CDR1 Peptide, CDR2 Peptide, and CDR3 Nucleotide, etc. columns are hidden by default. To clear the table, click the Clear Table button found in the table menu. It is possible to right click a cell in the table and copy the contents. An option will appear to allow you select the field you desire to copy. The data from this table can also be exported as covered later in this document under the "Export Data" section.

If there is not enough space in a column to display a cell's content, double-click on the column header's right divider in order to fully expand the column's width to fit its cells' contents.

Selected Unique CDR3s						
Well	Sample Name	Joined Nucleotide	CDR3 Peptide	V	D	J C +
B05	Sample BC03	ctctccctggagttcagacgtgtgctcttccgatcat	ASGQGNYGYT	hTRBV3-1	hTRBD1	hTRBJ1-2 hTRBC1
B05	Sample BC03	gacgcagagctgcaaacctgccttgtaatacactctac	IVRDNNNDMR	hTRAV26-1	*	hTRAJ43 hTRAC

Selected Unique CDR3s							
Well	Sample Name						+
B05	Sample BC03	ctctccctggagttcagacgtgtgctcttccgatcattacgacaagtcattaaatgtgaacaaaatctgggcatgatactatgtattggat					
B05	Sample BC03	gacgcagagctgcaaacctgccttgtaatacactctaccatcagtggaatgagatgtgtattggatcgacagattcactcccagggGCCACA					

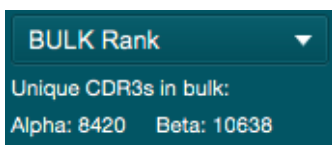


Analyzing a Project

Access the toolbox in order to begin an analysis on an iPair™ project. If the Analysis tab of the toolbox is not already open, click the Analysis button found above the plate.

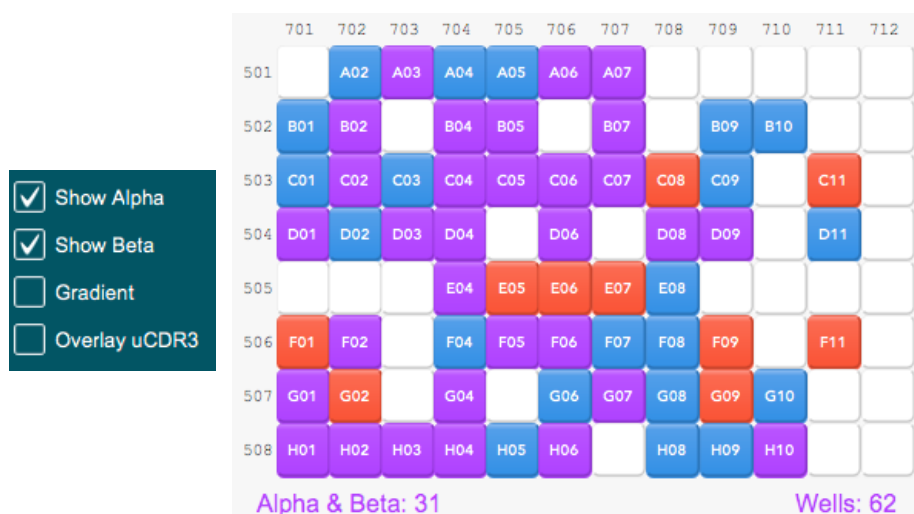


Under the Analysis tab of the toolbox, if the project contains bulk data, then the first drop-down menu contains the different bulks found within the project, and under it are the number of **Unique CDR3s** found in the bulk. Compare the **Bulk ranks** found in the tables to the total number of **Unique CDR3s** found in the bulk to help determine a **uCDR3's** significance.



An Alpha **uCDR3** with rank of 84 out of 8,420 **uCDR3s** is about as significant as a Beta **uCDR3** with a rank of 106 out of 10,638 **uCDR3s**.

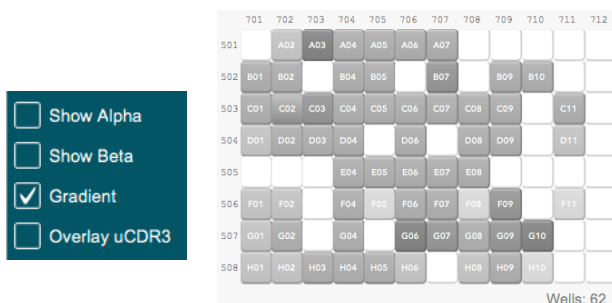
Below the bulk section is a group of checkboxes labeled Show Chain A, Show Chain B, Gradient, and Overlay uCDR3, where 'A' and 'B' in Show Chain A and Show Chain B will be replaced by whatever chains are found in the selected project (for example, TRG or TRD for gamma/delta or IGH or IGKL for heavy and light). By checking either Show Chain A or Show Chain B, the plate will change colors based on which wells have the chosen chain. Choosing both Show Chain A and Show Chain B will display three colors in the plate: a color for wells containing only chain A, a color for wells containing only chain B, and a color for wells that contain both chain A and chain B. These colors may be changed at any time. For the below example, blue represents **Alpha** only, red represents **Beta** only, and purple represents **both Alpha and Beta** were found in that well.





Show Gradient Feature

By checking **Show Gradient**, the wells will be colored based on their read depth by default. The gradient is calculated by the **Read Counts** of the uCDR3 in the well, which is typically correlated with amplification efficiency and library pooling. This gradient metric can be changed later. Wells with more sequencing reads will be darker, while wells with less will be lighter. Below is an example of **Show Gradient** being checked, while **Show Chain A** and **Show Chain B** checkboxes are left unchecked. In this example, we can tell that wells A03, G06, and G10 have relatively high read counts, while wells F05, F08, F11, and H10 have relatively low read counts.



Overlay uCDR3 Feature

Next is the **Overlay uCDR3** checkbox, which will not initially change the plate when selected; however, two icons will appear above the top two tables on the right. In this case, the **Alpha** table is represented by a circle shape, and the **Beta** table is represented by a diamond shape.

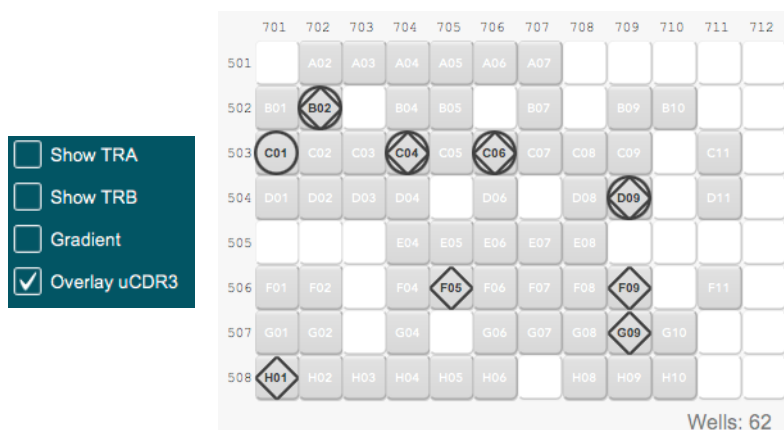
Alpha C04 Sample BC03		Beta C04 Sample BC03	
CDR3 Peptide	Read Count +	CDR3 Peptide	Read Count +
AMRGGTSYGKLT	18758	SVTGTSGSNNEQF	7814
AMSENNARLM	140	ATSDLGAGPLTDTQY	167

Double-click on a **uCDR3** in the tables in order to overlay the selected **uCDR3**. Doing so while the **Overlay CDR3** checkbox is checked will cause any instance of the selected **uCDR3** to be marked in the tables, as shown below.

○ Alpha C04 Sample BC03		◇ Beta C04 Sample BC03	
CDR3 Peptide	Read Count +	CDR3 Peptide	Read Count +
AMRGGTSYGKLT	18758	SVTGTSGSNNEQF	7814
AMSENNARLM	140	ATSDLGAGPLTDTQY	167



In addition to this, wells containing the selected **uCDR3's** peptide will now have their respective symbols overlaid on top. Wells containing the selected chain A **uCDR3's** peptide will have a circle overlay, wells containing the selected chain B **uCDR3's** peptide will have the diamond overlay, and wells containing both the selected chain A **uCDR3's** peptide and the selected chain B **uCDR3's** peptide will have both a circle and a diamond overlay. This allows for a quick and easy analysis of receptor pairs that show up multiple times on a plate.



In the above example, well C01 contains the selected Alpha **uCDR3** peptide, wells F05, F09, G09, and H01 contain the selected Beta **uCDR3** peptide, and wells B02, C04, C06, and D09 contain both the selected Alpha **uCDR3** peptide and the selected Beta **uCDR3** peptide.

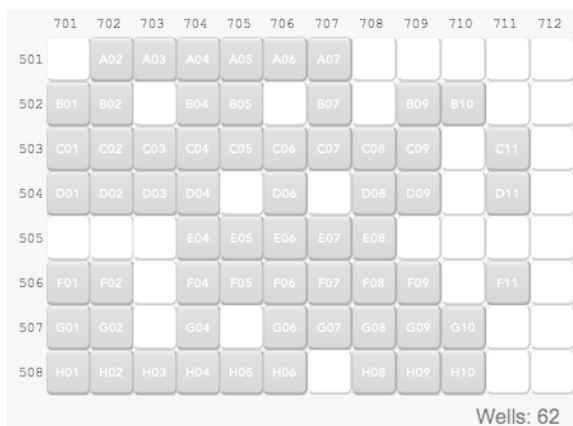
To hide a selected **uCDR3**, click on the overlay icon above the table. To unhide a selected **uCDR3**, click on the overlay icon again, or select another **uCDR3**. Below is an example how the selected Beta **uCDR3's** peptide becomes hidden.

Alpha C04 Sample BC03		Beta C04 Sample BC03	
CDR3 Peptide	Read Count +	CDR3 Peptide	Read Count +
AMRGGTSYGKLT	18758	SVTGTSGSNNEQF	7814
AMSENNARLM	140	ATSDLGAGPLTDTQY	167



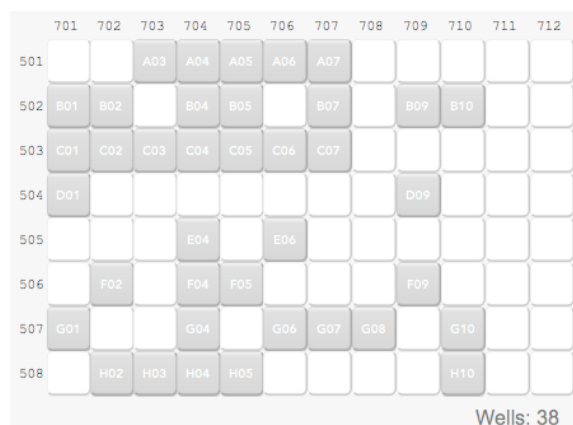
Below the checkboxes are a group of radio buttons for **Read Count** and **Rank**. Having **Read Count** selected indicates that the plate will display well significance based on the **uCDR3's Read Count**. If the **Gradient** checkbox is unchecked while Read Count is selected, then wells containing **uCDR3s** with any **Read Count** are displayed, as shown below. If the **Gradient** checkbox is checked, then wells containing higher relative **Read Counts** will be darker, while wells containing lower relative **Read Counts** will be lighter. The counter below the plate will count the number of wells containing **Read Counts**.

Read Count
 Rank



Having **Rank** selected indicates that the plate will display well significance based on the **uCDR3's Bulk Rank**. If the **Gradient** checkbox is unchecked while Rank is selected, then wells containing **uCDR3's** with any Rank (not including N/A, which means it wasn't found in the Bulk) are displayed in the plate, as shown below. If the **Gradient** checkbox is checked, then wells containing higher relative **Ranks** will be darker, while wells containing lower relative **Ranks** will be lighter. The counter below the plate will count the number of wells containing **Ranks** other than N/A.

Read Count
 Rank





Bulk Cell Comparison Feature

Sometimes during a project, repeat libraries for the bulk sample may have been sequenced or the bulk sample for other cell types from the same sample were created. To change which bulk the uCDR3's are being compared to, use the bulk drop-down menu found near the top of the toolbox.



Color Scheme Feature

Underneath the Plate Display section is **Color Scheme**. Click on **Color Scheme** in order to fully expand the section if it is not already expanded. There is a drop-down menu that represents what color scheme is currently selected. Changing the color scheme will change the colors in the plate when **Show Chain A** checkbox or **Show Chain B** checkbox are checked, and will also change the colors in the tables. Below the drop-down menu is a legend displaying which colors represent which chains. Below is an example of the default color scheme.

The screenshot shows a dropdown menu for the 'Color Scheme' set to 'Twilight Sky'. The legend below the menu shows three categories: Alpha (blue circle), Alpha & Beta (purple circle), and Beta (orange circle). To the right is a 9x12 grid representing a plate layout. The columns are labeled 701-712 and the rows are labeled 501-508. Wells are color-coded according to the legend: Alpha (blue), Alpha & Beta (purple), and Beta (orange). The grid shows a pattern of wells, with some wells empty. At the bottom of the grid, it says 'Alpha & Beta: 31' and 'Wells: 62'.

	701	702	703	704	705	706	707	708	709	710	711	712
501		A02	A03	A04	A05	A06	A07					
502	B01	B02		B04	B05		B07		B09	B10		
503	C01	C02	C03	C04	C05	C06	C07	C08	C09		C11	
504	D01	D02	D03	D04		D06		D08	D09		D11	
505				E04	E05	E06	E07	E08				
506	F01	F02		F04	F05	F06	F07	F08	F09		F11	
507	G01	G02		G04		G06	G07	G08	G09	G10		
508	H01	H02	H03	H04	H05	H06		H08	H09	H10		

Alpha & Beta: 31 Wells: 62



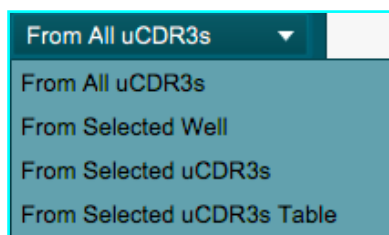
Exporting Data

In order to export data from an iPair project, click the **Export** tab found at the top of the toolbox. If the Export tab is not available, going to the **File** menu and selecting Export will open up the export options.



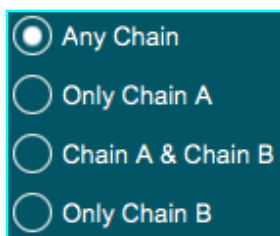
Under the **Export** tab of the toolbox, you can customize exactly what data gets exported into a CSV file. A CSV file is used for spreadsheets, and can be opened in a most spreadsheet software, like Excel.

At the top of the toolbox is a drop-down menu that has **From All uCDR3s** selected by default.



The **From All uCDR3s** option indicates that all **uCDR3s** will be exported to the CSV file. The **From Selected Well** option indicates that all **uCDR3s** found in the currently selected well, i.e. the **uCDR3s** appearing in the top two tables on the right, will be exported to the CSV file. The **From Selected uCDR3s** option will export all instances of the **uCDR3s** selected in the Overlay feature. Finally, the **From Selected uCDR3s Table** indicates that all **uCDR3s** found in the Selected uCDR3s table will be exported. All of the above is assuming that there are no other filters.

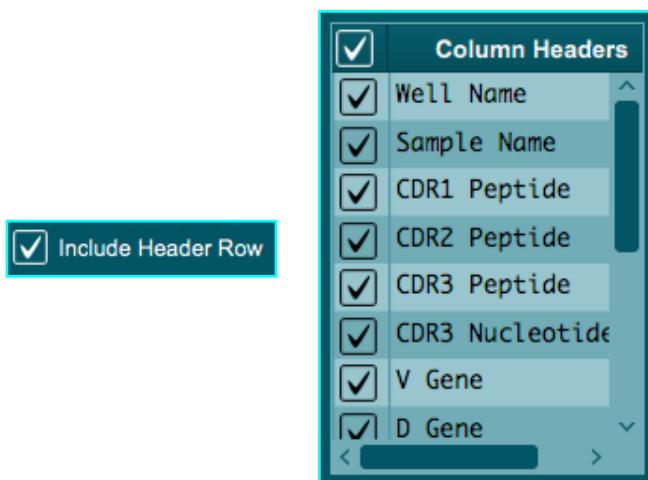
Next, there is a group of radio buttons that will filter the **uCDR3s** based on chain. By default, the **Any Chain** option is selected, which indicates that **uCDR3s** will be exported to the CSV file regardless of their chain. Selecting the **Only Chain A** or **Only Chain B** options, where 'A' and 'B' represent the chains found in the project, will have only the selected chain exported in the CSV file. The **Chain A & Chain B** option will only export **uCDR3s** from wells where both chains are present.





The Header Row

Beneath that is the option to include the **Header Row**. The **Header Row** is the first row of the CSV file which contains the name describing each column. By default, this option is checked. After **INCLUDE HEADER ROW**, is a table containing all of the different columns that can appear in the CSV file. Any column header that was not selected will not appear in the CSV file.



Finally, when everything is set, click the **Export to CSV** button in order to export the CSV file to your desired location. The following data was exported using the example project with the following settings: From Selected Well (well D09), Only Chain Alpha, included Header Row, and columns Well Name, CDR3 Peptide, V, D, J, and C included.

Well Name	CDR3 Peptide	V	D	J	C
D09	ALGGLNQGGKLI	hTRAV38-1	*	hTRAJ23	hTRAC
D09	AMSENNNARLM	hTRAV12-3	*	hTRAJ31	hTRAC



iRepertoire, Inc.

Contact Information and Reporting

Contact Information

Please be sure to include your name, organization, and a detailed description of the request and/or error you are encountering. If requesting raw, demultiplexed reads, please provide a specific Project and Study Name including the date submitted.

Office Hours: Monday - Friday, 8:00 AM to 5:00 PM CST (Central Standard Time)

Telephone/Fax: 1 (256) 327-0948

Email: info@irepertoire.com

Bug/Error Reports & Suggestions

Bugs/Errors: While using the iPair Analyzer™, if you find bugs or errors that occur in the software, please report it. A bug or error would be the iPair Analyzer™ behaving in unexpected ways, and may result in an alert box with an associated code. Please contact us with information about the bug/error that you discovered. In order to help find the issue the fastest, please include what actions you took in order to find the bug or error, and the code found in the alert box (if available).

Suggestions: We would like for the iPair Analyzer™ to give the best user experience as possible, so if you have any suggestions for improvements we would love to hear from you. Please contact us with information about your suggestions, and your ideas may be considered in future versions of the iPair™ and the iPair Analyzer™.