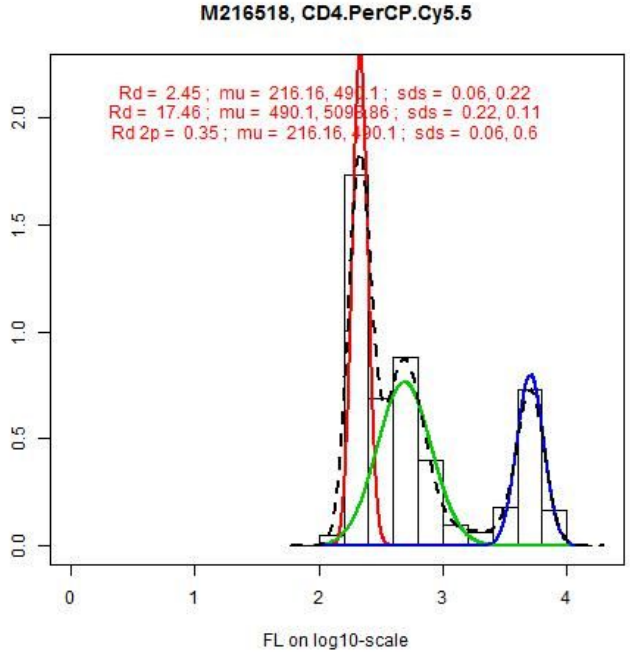
HOW TO SETUP MULTICOLOR ASSAYS IN CHIPCYTOMETRY

1. Assay Development

Prior to establishing the multicolor antibody cocktail, for each marker the optimal epitope-dye combination and staining procedure (antibody dilution, buffer, incubation time and temperature) has to be established. This is called assay development.
The objective measure of having found a good epitope-dye combination and staining protocol is a high signal-to-background ratio. A good approximation of the signal-to-background ratio can be calculated by using Fisher's discrimination ratio (FDR)[1] given that the marker is really separating the cells into one or more positive and a negative subpopulations. The figure below shows the FDRs for human CD4-PerCP-Cy5.5 stained on human PBMC. The green peak is CD4 on monocytes having a FDR of 2.45, the blue peak is CD4 on T-cells with an FDR of 17.46.



FDR calculation can be ordered within the Datawizard of the ZKWApp. The job type is 'DiscRatio-SingleChip'. Once the job is finished, double-clicking on the job will open a folder containing JPG-images (use the ones containing the term 'mixmdl') of the FDR data visualisation for each marker.

[1] K. Fukunaga. Introduction to Statistical Pattern Classification. Academic Press, San Diego, California, USA, 1990.

1.1 Selection of suitable clones

If you are using Chipcytometry with biobanked samples, you have to work with clones that recognize PFA-fixed epitopes (>80% of flow cytometry antibodies for surface markers, 100% of intracellular markers for flow cytometry, 100% of all IHC clones).

Here is our suggested strategy to find clones that you could use:

1. The best point to start from is to look at our validated panels on the Zellkraftwerk webpage (http://www.zellkraftwerk.com/ZKWAntibodies.phtml#Panels).
2. If you cannot find an appropriate antibody there, you might write an email to support@zellkraftwerk.com to ask if we have something in the pipeline.
3. You can browse the internet for IHC (immunhistochemistry)-tested clones that are available in a dye-labelled format.

However, if you are doing live-cell assays or live-stainings, you can use any available flowcytometry-validated antibody for surface markers.

1.2 Selection or generation of an appropriate positive control

Very important is the selection of a suitable positive control, e.g., a good control for costimulatory molecule PD-1 is PHA-stimulated PBMC. For each marker, the best positive control should be selected.

1.3 Selection of suitable colors for the selected clones

The following filter-dye combinations have been tested to robustly work with our filtersets. The most sensitive filterset is FS560, so we recommend to use the other filtersets for lineage markers and FS560 for markers where you aim to measure MFI with high sensitivity.

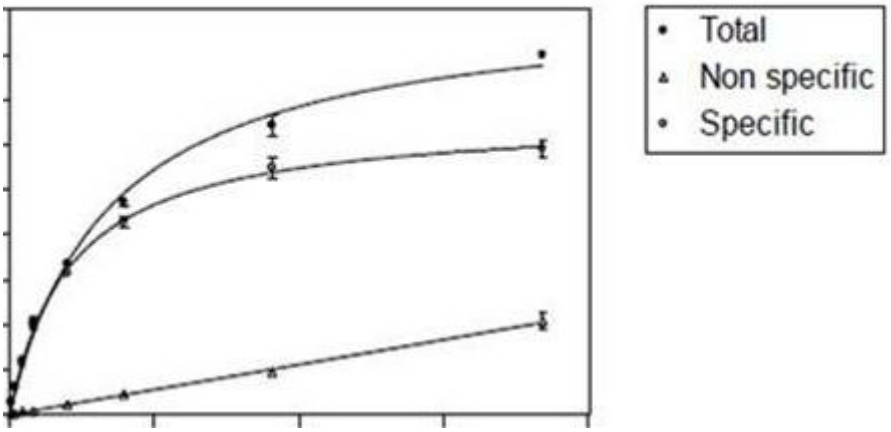
Filterset	Dye
FS395	Brilliant Ultraviolet 395
FS421	Brilliant Violet 421
FS488	Alexa-Fluor 488
FS560	PE
FSPerCP	PerCP-Cy5.5

Please note: New dyes have to be tested prior to use, since it is known that dye-labelling influences the binding properties of antibodies significantly.

[1] K. Fukunaga. Introduction to Statistical Pattern Classification. Academic Press, San Diego, California, USA, 1990.

1.4 Titration of the antibody

Since unspecific binding increases in a linear way while the specific binding shows an asymptotic behavior, too-high and too-low concentration of the antibody will result in low FDR. The figure below shows typical specific and non-specifc binding curves of a titration experiment.



To determine the optimal dilution of an antibody, a titration curve should be generated using at least 4 dilutions (e.g. 1:1000, 1:400, 1:100, 1:30) on separate chips. Calculate the FDR for each titration step - FDR reflects the distance between non-specific and specific staining.

Please note: Antibody vendors of RUO reagents (research use only) cannot guarantee that between different lots of the same antibody, quantity of dye labelling is the same. Therefore, it is recommended to repeat the titration .

2. Panel Optimisation

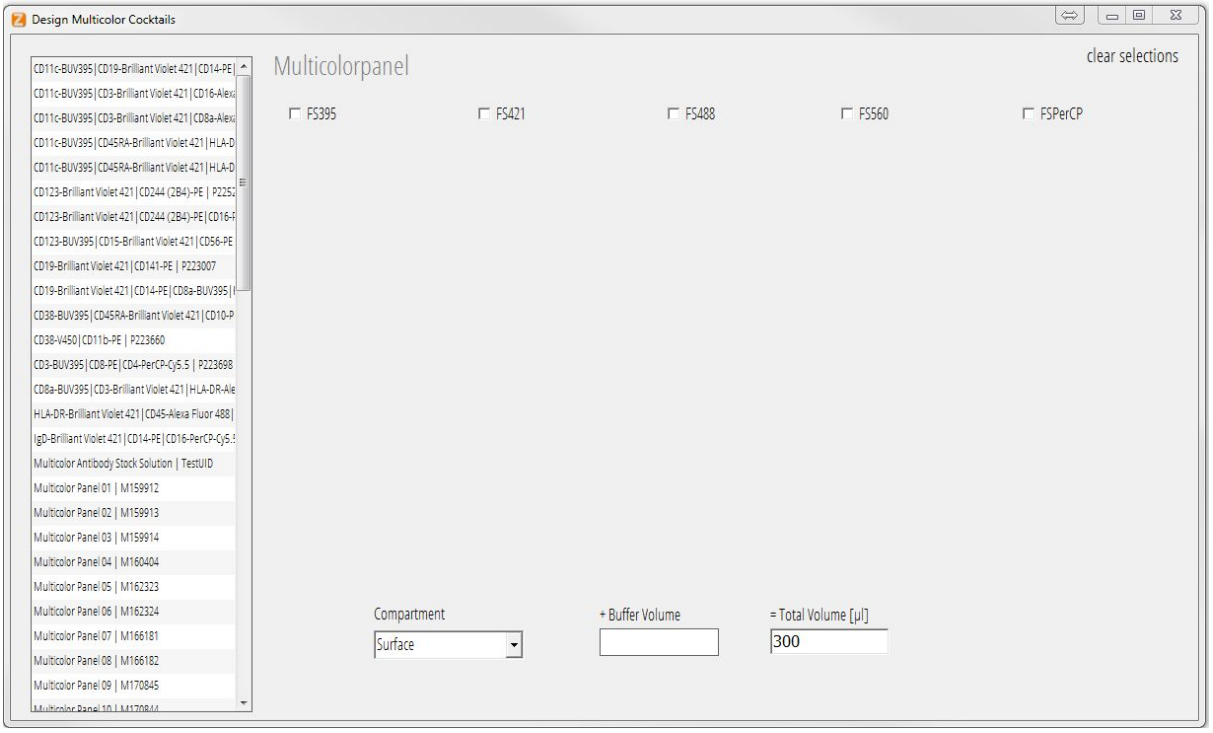
Although after assay development each antibody should work nicely if stained as single-plex assay, building multiplex cocktails requires test of the staining quality of each antibody if stained within the cocktail. Again, FDR (Fisher's discrimination ratio) is used as parameter of choice to control the quality of the stain. The FDR of each antibody stained within the multiplex-cocktail should be similar to the FDR of the same antibody when stained single-plex. To stain multicolor cocktails on Chipcytometry systems, generate the cocktail within ZKWApp as described in appendix A.

[1] K. Fukunaga. Introduction to Statistical Pattern Classification. Academic Press, San Diego, California, USA, 1990.

APPENDIX

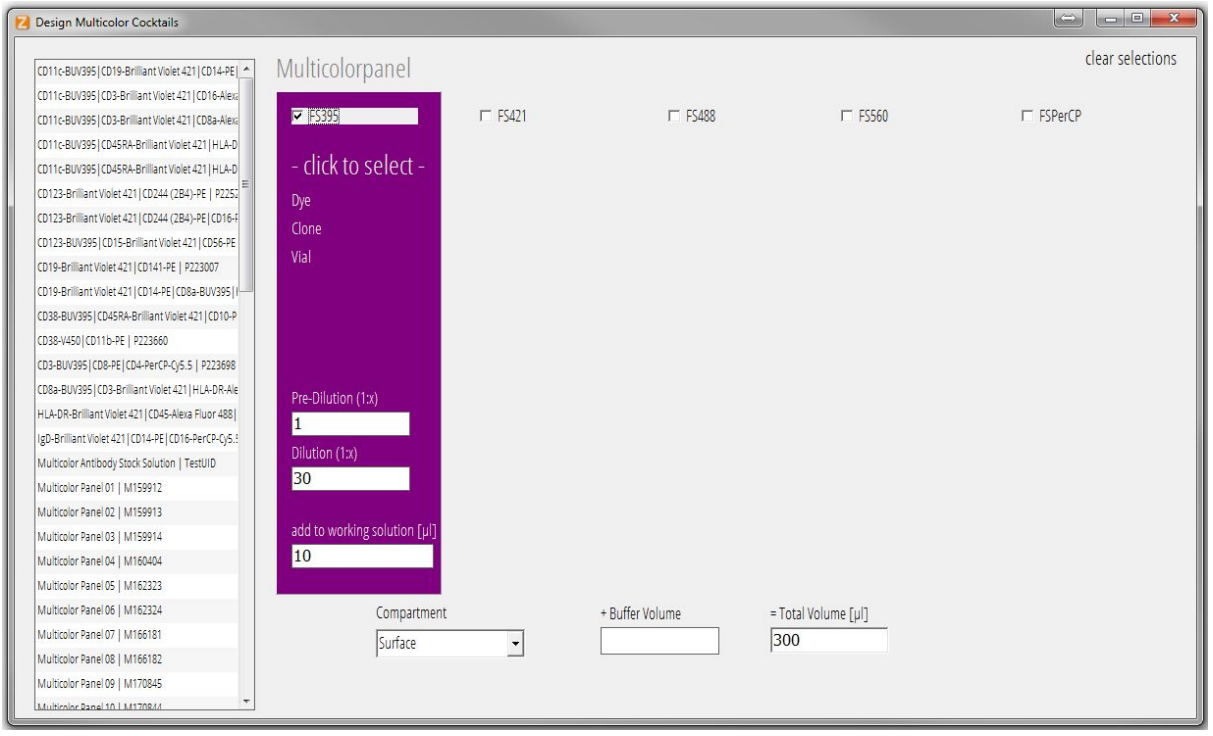
A. Generate Multicolorcocktail in ZKWApp

Multicolor Cocktails are generated for subsequent use in Chipytometry within the Multicolor Cocktail Shaker (ZKWApp → Stock Manager → Action → Design multicolor cocktail.

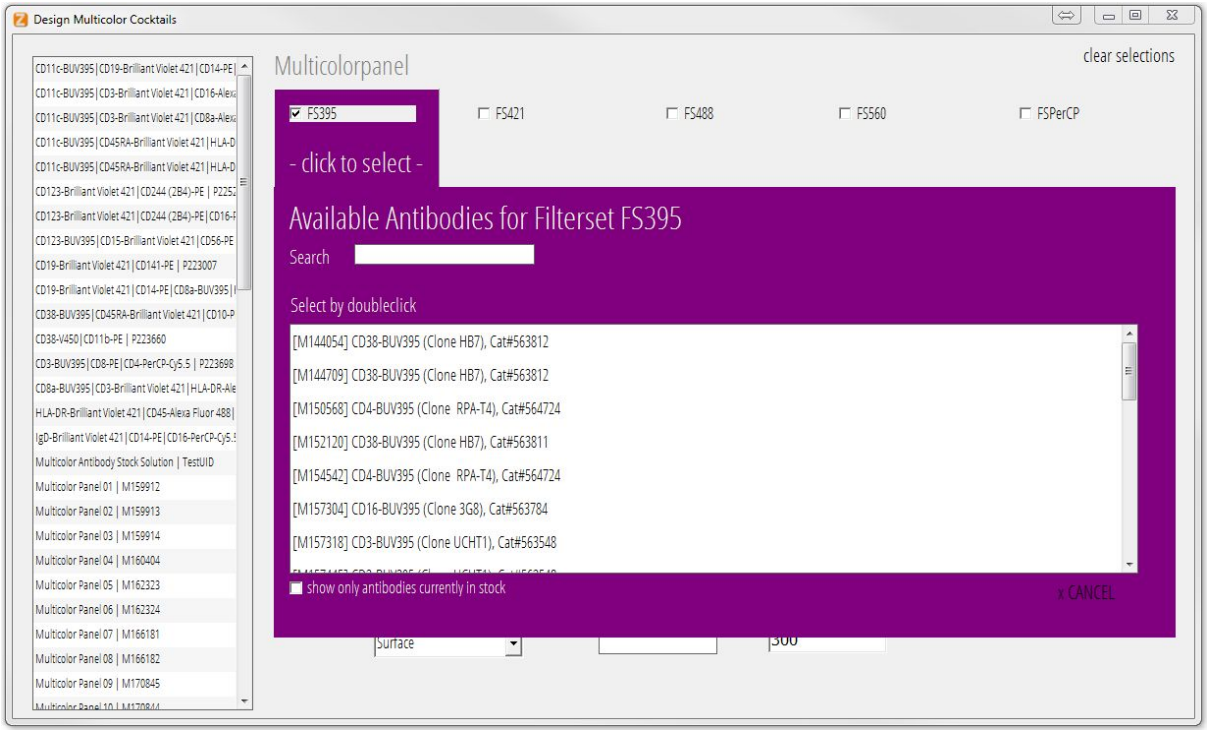


On the left, all available cocktails are displayed and can be selected by left-click.
To generate a new cocktail, click on the checkbox of the respective filterset.

[1] K. Fukunaga. Introduction to Statistical Pattern Classification. Academic Press, San Diego, California, USA, 1990.

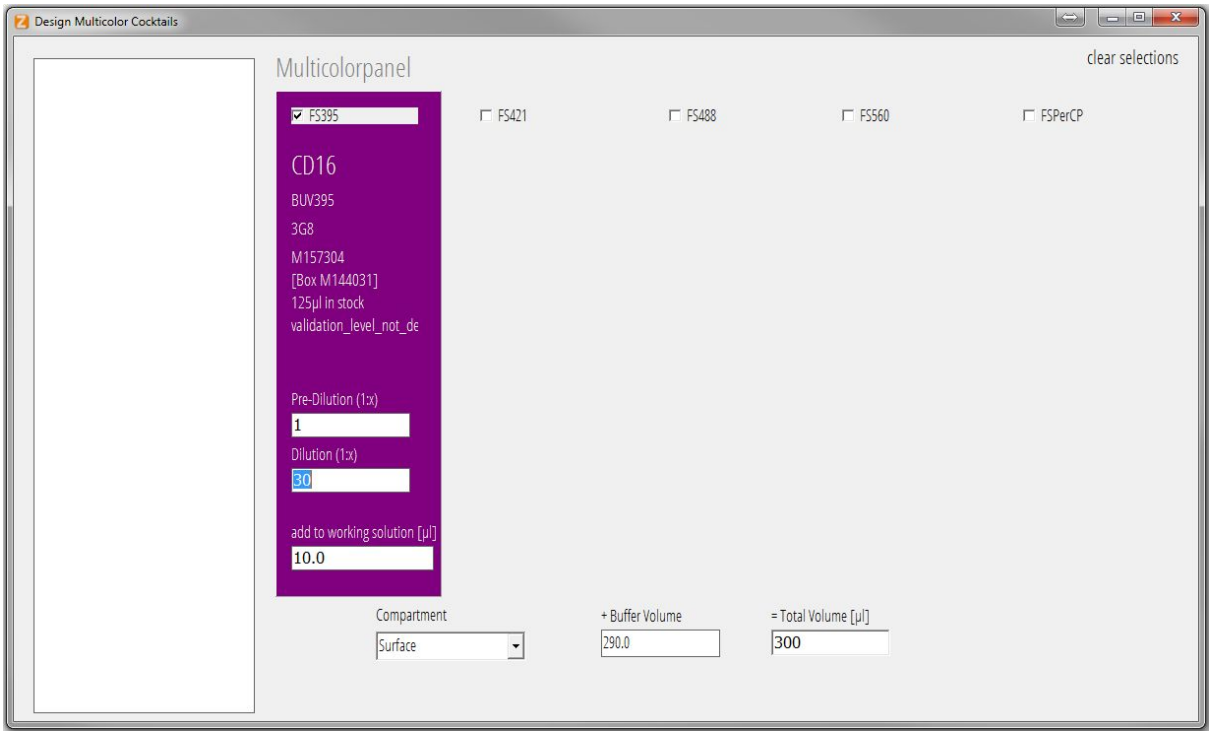


Next, click on '- click to select -' to display all available antibodies for this filterset in your stock (present and already discarded vials).



Using the search box, you can further narrow down the list. Double-click on the desired marker.

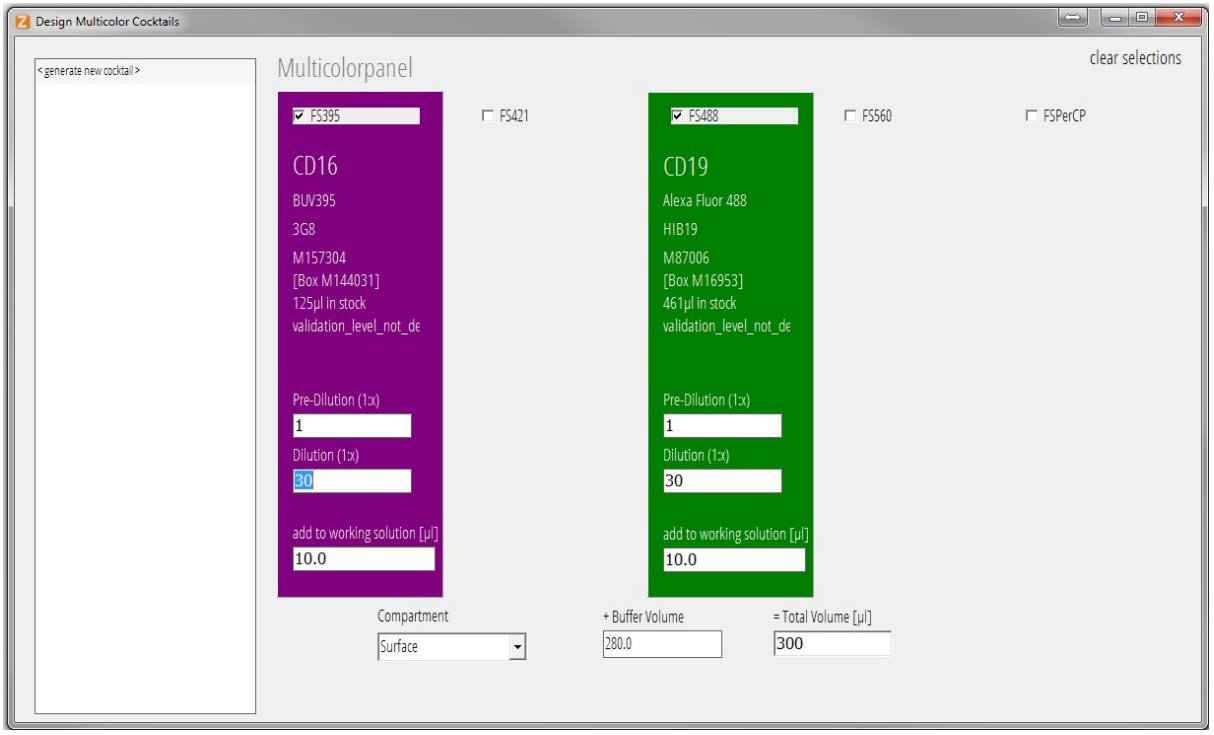
[1] K. Fukunaga. Introduction to Statistical Pattern Classification. Academic Press, San Diego, California, USA, 1990.



The app looks up pre-dilutions and dilutions already established with this marker. Since dilutions might differ between single-plex and multi-plex use of each antibody, dilution and pre-dilution can be changed for each antibody here and will be stored specifically for each cocktail.
If a higher dilution is used with an antibody, it might be preferable to use a serial dilution (e.g., 1:10 -> 1:40 for a 1:400 dilution) to minimize bias due to pipetting errors. In this case, a pre-dilution can also be attributed to each marker.

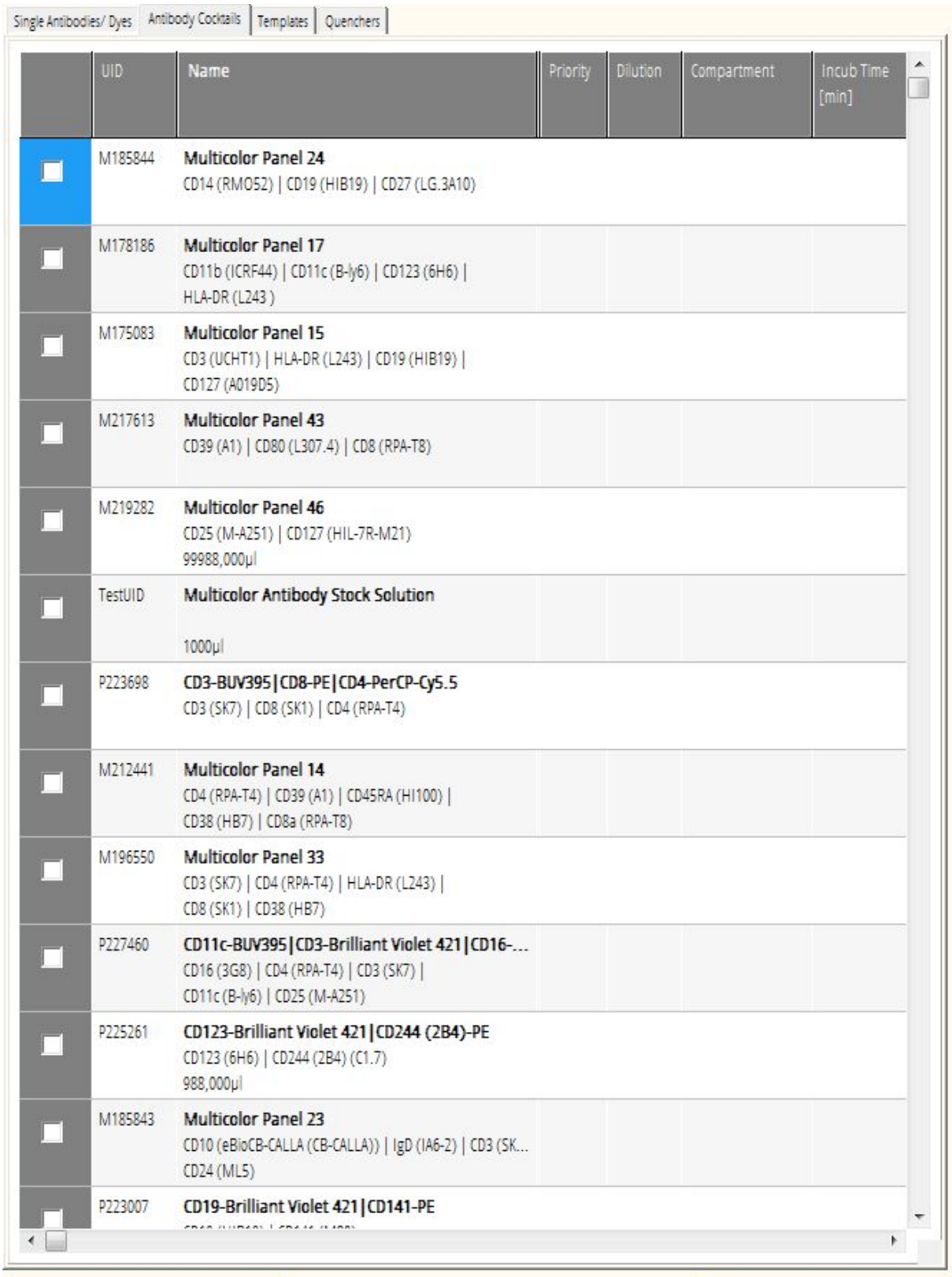
Select antibodies for each filterset, you can choose an antibody. The app checks if multicolor-cocktail with these antibodies already exist (shown in the list on the left). If now such a cocktail does not exist, you can generate a new one by clicking on <generate new cocktail>:

[1] K. Fukunaga. Introduction to Statistical Pattern Classification. Academic Press, San Diego, California, USA, 1990.



You are offered to change the name of the new cocktail, and that's it. You now can order the Cocktail in the Project Manger → Action → Order Markers within the 'Antibody Cocktails' tab:

[1] K. Fukunaga. Introduction to Statistical Pattern Classification. Academic Press, San Diego, California, USA, 1990.



[1] K. Fukunaga. Introduction to Statistical Pattern Classification. Academic Press, San Diego, California, USA, 1990.