

PRODUCT INSERT

Instrument Compatibility: Cellaca[®] PLX

Cellaca[®] PLX, anti-human CD3 KB520 / CD4 PE / CD8 APC Total Cell Kit

Part number:	CSK-A0026-1	CSK-A0026-2
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Test number:	25 Tests	100 Tests
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Storage: 4°C

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1. Introduction

1.1. Description

CD3/CD4/CD8 surface marker reagents with a total dye (Hoechst) are designed for researchers interested in acquiring data on three surface marker populations, as each patient and cell line derived sample can be unique. The Cellaca® PLX provides users with fluorescent and bright field images of their Hoechst and CD3/CD4/CD8 stained cells. Data can be automatically exported from PLX Matrix software into FCS Express software templates with preset gates for rapid data analysis.

1.2. Kit contents

This kit assesses CD3/CD4/CD8 populations with Hoechst as the total dye on the Cellaca® PLX. The anti-human CD3 antibody is conjugated with KIRAVIA Blue 520™¹, anti-human CD4 with PE, and anti-human CD8 with APC fluorophores. See table below for kit components and corresponding surface markers with their respective isotype controls.

Cellaca® PLX Assay	Reagents	Catalog Number	Number of Tests
PLX.5_3SM+Total___ CD3-KB + CD4-PE + CD8- APC + Hoechst	KIRAVIA Blue 520™ anti-CD3 (UCHT1) (Component A) PE anti-human CD4 (RPA-T4) (Component B) APC anti-human CD8 (RPA-T8) (Component C) KIRAVIA Blue 520™ Mouse IgG1 Isotype (Component D)	CSK-A0026-1	25
	PE Mouse IgG1 Isotype (Component E) APC Mouse IgG1 Isotype (Component F) Hoechst 33342 (Component G)	CSK-A0026-2	100

1.3. Required Materials

- Cellaca® PLX image cytometer (Revvity)
- Revvity-provided Laptop with Matrix 5.0 Software or above (pre-installed)
- FCS Express software (pre-installed on Revvity-provided laptop) with dongle/license
- Cellaca® PLX Low Fluorescence Slides (Cat. # CHM2-ACR)
- Cellaca® PLX slide holder
- Reagents provided in kit CSK-A0026
- 1X Phosphate Buffered Saline (PBS)
- Microcentrifuge tubes
- Cell culture media
- Cells or PBMC's

¹ KIRAVIA Blue™ 520 is a trademark of Sony. This product is subject to proprietary rights of Sony and is made and sold under license from Sony Corporation.

2. Staining Procedure for CD3 KB520 / CD4 PE / CD8 APC with Hoechst

Cellaca® PLX Assay	Reagents	Catalog Number	Number of Tests
PLX.5_3SM+Total__ CD3-KB + CD4-PE + CD8- APC + Hoechst	KIRAVIA Blue 520™ anti-CD3 (UCHT1) (Component A)	CSK-A0026-1	25
	PE anti-human CD4 (RPA-T4) (Component B)		
	APC anti-human CD8 (RPA-T8) (Component C)		
	KIRAVIA Blue 520™ Mouse IgG1 Isotype (Component D)	CSK-A0026-2	100
	PE Mouse IgG1 Isotype (Component E)		
	APC Mouse IgG1 Isotype (Component F)		
	Hoechst 33342 (Component G)		

For each sample:

- For a single sample, prepare 2 microcentrifuge tubes with 1×10^6 PBMCs/cells each
NOTE 1: For 1×10^6 cells, take 1 mL of 1×10^6 cells/mL
NOTE 2: For multiple samples, prepare 2 tubes each
- Label tubes, accordingly, one for staining with antibodies (**Ab**) and one for isotype control (**Ctrl**) staining for each distinct sample
- Centrifuge cells at 1200 rpm for 5 minutes
- Remove supernatant from all tubes avoiding cell pellets
- Dilute Hoechst 33342 by adding 1 μ L of **Hoechst 33342** (Component G) to 19 μ L 1X PBS
NOTE: 1:20 dilution for 1 mM working stock
- Resuspend the cell pellets from all tubes in 90 μ L of cell culture media
NOTE: Staining in 1X PBS results in dimmer signal
- For staining cells in **Ab tubes**, add the following, and mix well:
 - 5 μ L of **CD3 KB520** (Component A)
 - 5 μ L of **CD4 PE** (Component B)
 - 5 μ L of **CD8 APC** (Component C)
 - 1 μ L of Hoechst 33342 working stock (diluted from step 5)

NOTE: If testing 2-4 samples, we recommend creating a master mix, according to the table below. After adding all components to form the master mix, add 15 μ L of the master mix stain to each **Ab tube** and mix well.

	2 samples	3 samples	4 samples
CD3 KB520 (Component A)	10 μ L	15 μ L	20 μ L
CD4 PE (Component B)	10 μ L	15 μ L	20 μ L
CD8 APC (Component C)	10 μ L	15 μ L	20 μ L
Hoechst working stock (Diluted from step 5)	2 μ L	3 μ L	4 μ L

8. For staining cells in **Ctrl tubes**, add the following, and mix well:

- 5 µL of **IgG1 KB520** (Component D)
- 2.5 µL of **IgG1 PE** (Component E)
- 1.2 µL of **IgG1 APC** (Component F)
- 1 µL of Hoechst 33342 working stock (diluted from step 5)

NOTE: If testing 2-4 samples, we recommend creating an isotype control master mix, according to the table below. After adding all components to form the isotype control master mix, add 9.5 µL of the isotype control master mix stain to each **Ctrl tube** and mix well.

	2 samples	3 samples	4 samples
IgG1 KB520 (Component D)	10 µL	15 µL	20 µL
IgG1 PE (Component E)	5 µL	7.5 µL	10 µL
IgG1 APC (Component F)	2.5 µL	3.7 µL	5 µL
Hoechst working stock (Diluted from step 5)	2 µL	3 µL	4 µL

9. Incubate all tubes in the dark for 10 minutes at 37 °C

10. To each tube, add 200 µL of 1X PBS and mix well

11. Centrifuge cells at 1200 rpm for 5 minutes

12. Remove supernatant from each tube avoiding cell pellets

13. Resuspend each cell pellet in 100 µL of cell culture media

NOTE: Resuspension in 1X PBS results in dimmer signal

14. Mix samples thoroughly by pipetting up and down a few times

15. Load 15 µL of sample from **Ab tube** into side A of the slide

NOTE 1: Loading samples in wrong side results in incorrect sample output in FCS Express

NOTE 2: Repeat for any additional samples prepared

16. Load 15 µL of sample from **Ctrl tube** into side B of the slide

NOTE: Repeat for any additional samples prepared

17. To image replicates from the same sample, load another slide following steps 15 and 16

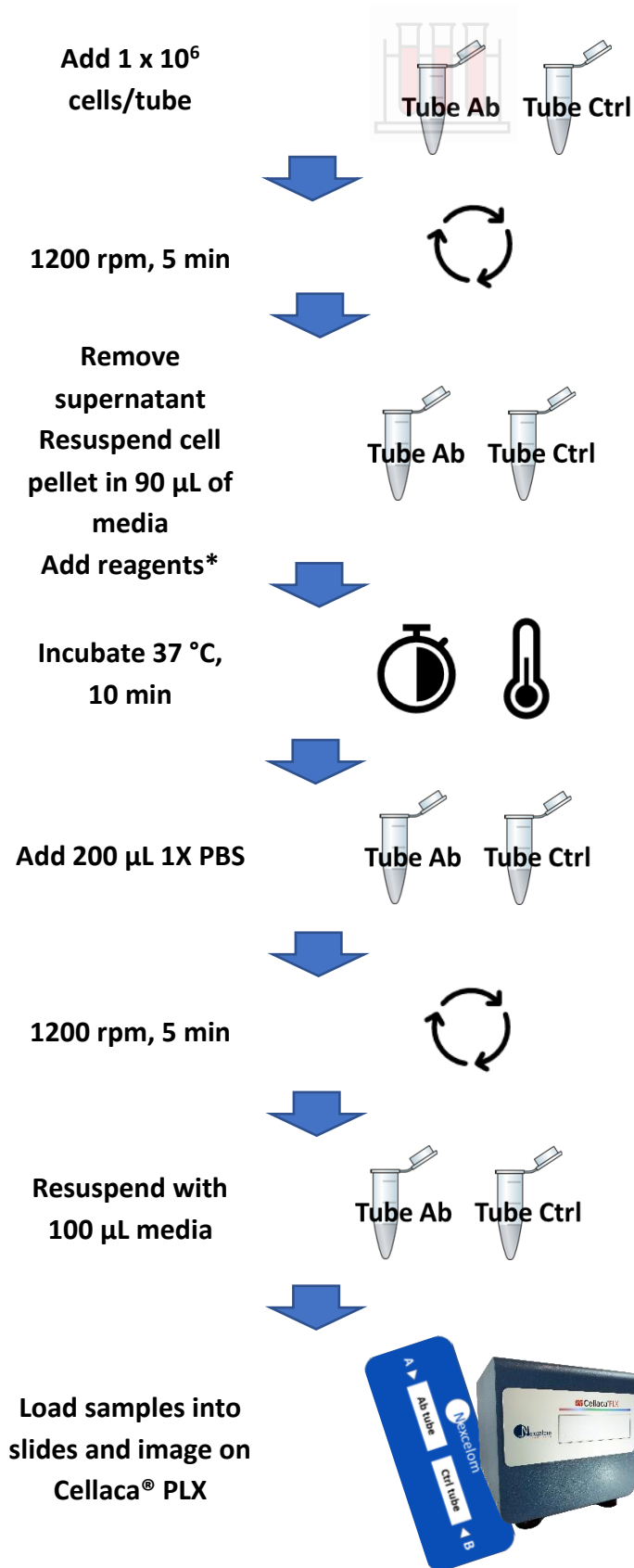
18. Place slides into slide holder, with side A at the top, as shown in the diagram

NOTE: Notched edge of the slide holder is the top left

19. Proceed to section 4 for image and data acquisition



3. Expert User Quick Guide – CD3 KB520 / CD4 PE / CD8 APC with Hoechst



* Dilute **Hoechst** 1:20 in 1X PBS

* For **Ab tubes:**

	Samples			
	1	2	3	4
CD3 KB520	5 µL	10 µL	15 µL	20 µL
CD4 PE	5 µL	10 µL	15 µL	20 µL
CD8 APC	5 µL	10 µL	15 µL	20 µL
Hoechst	1 µL	2 µL	3 µL	4 µL

Add 15 µL of the master mix to each tube

* For **Ctrl tubes:**

	Samples			
	1	2	3	4
IgG1 KB520	5 µL	10 µL	15 µL	20 µL
IgG1 PE	2.5 µL	5 µL	7.5 µL	10 µL
IgG1 APC	1.2 µL	2.5 µL	3.7 µL	5 µL
Hoechst	1 µL	2 µL	3 µL	4 µL

Add 9.5 µL of the master mix to each tube

4. Cellaca® PLX Image and Data Acquisition

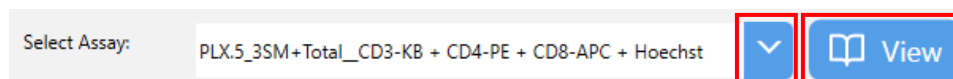
4.1. Initiate software and load samples

- 4.1.1. Start the **Matrix** software by double-clicking the icon on the desktop of the operating computer
- 4.1.2. Software will direct you to the **Acquire, Setup** tab by default
- 4.1.3. Click **Eject** to open the instrument stage
NOTE: Button located at the top of the Acquire tab
- 4.1.4. Place the slide holder containing slide(s) into the ejected stage
NOTE: Align the notched edge of the holder in the upper left corner
- 4.1.5. Click the **Load** button to retract the instrument stage



4.2. Assay Selection

- 4.2.1. In **Setup Details**, type in a **Plate Name**
- 4.2.2. **Select Assay** from the dropdown



- 4.2.3. To edit or review assay settings, click the blue **View** tab to the right of the assay selection

NOTE: See Assay Settings, Cell Type Parameters, and Auto Export Data and Images sections in the Appendix for detailed information regarding assay, cell parameters, and report/export information, respectively.


4.3. Well Details and Assign Well Names

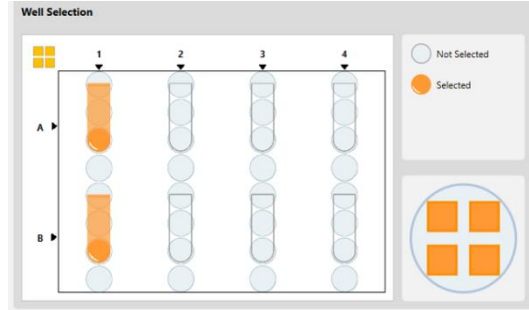
- 4.3.1. In **Well Details**:
 - 4.3.1.1. Select "4 Slides (CHM2-ACR)" as the **Plate Type**



4.3.2. In **Well Selection**, select the well(s) to be imaged

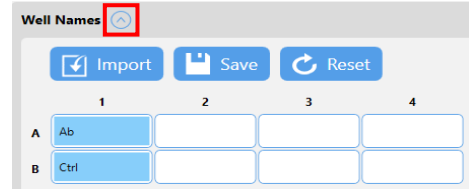
NOTE 1: Selected samples will turn orange.

NOTE 2: To select or clear multiple wells, click a well and hold/drag your mouse to encompass other wells. To select or clear all wells, click the  button.



4.3.3. To assign **Well Names**, click the downward facing arrow

4.3.3.1. Type in well/sample name(s)

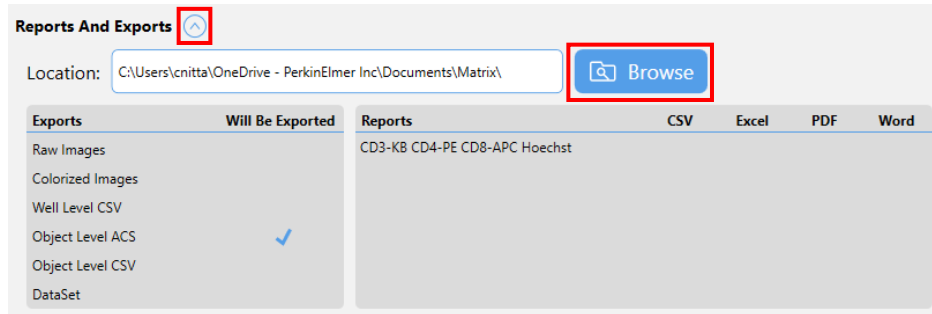


4.4. Reports and Exports

4.4.1. Click the downward facing arrow to open the reports and exports details

4.4.2. In **Location**, click on the browse button to select or create an export location.

NOTE: Images and data selected to be exported will have a blue checkmark

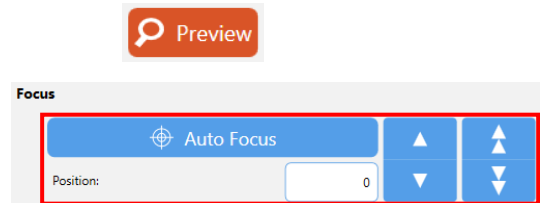


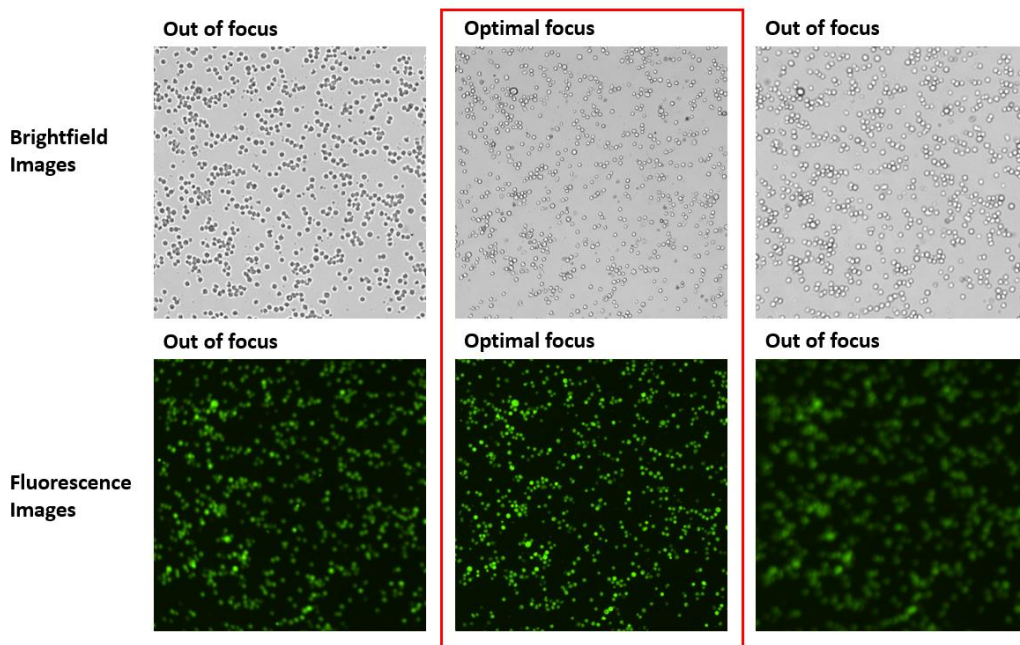
4.5. Preview Samples

4.5.1. Click **Preview** button to view the sample

4.5.2. In **Focus**, click **Auto Focus** to focus the sample in Brightfield for Channel 1

NOTE: If needed, manual focusing can be done using **double arrows** for coarse and **single arrow** for fine adjustments

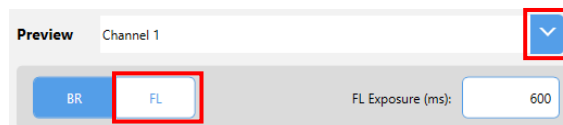




4.5.3. Once the sample is focused, click the **FL** button to preview Channel 1 fluorescence


4.5.3.1. Adjust exposure times as needed

NOTE: See *Recommended Surface Marker and Total Dye Exposure Times and Filter Pairs in the Appendix*



4.5.4. Select subsequent fluorescence channels using the **Preview** dropdown menu

4.5.5. Click the **FL** button to preview the fluorescence in each channel and adjust exposure times as needed

4.5.6. Click the **Count** button when ready to acquire and analyze samples 

4.6. FCS Express


4.6.1. FCS Express will automatically initialize and populate with data generated from this scan

4.6.2. In the data list, confirm that your samples in the File Name column are in the correct order according to the Tube column (Ex: object_A1.acs and object_B1.acs as Sample 1 and Sample 1 Isotype, respectively)

NOTE 1: *If samples are not in the correct order, use the up and down arrows to move them to the correct location.*

NOTE 2: *If samples are not in the correct order data will not be accurate.*

Data List



Iteration	Tube	File Name
1	1 (Sample 1)	object_A1.acs
	2 (Sample 1 isotype)	object_B1.acs
	3 (Sample 2)	object_A2.acs
	4 (Sample 2 isotype)	object_B2.acs

5. Additional Resources

5.1. Storage / Safety

Store each product at 4 °C, protected from light. Please consult the Safety Data Sheet for more safety information, found on www.revvity.com/cellcountingreagents.

5.2. Warranty

This product is for RESEARCH USE ONLY and is not approved for diagnostic or therapeutic use. Product is warranted to meet the specifications outlined in the Certificate of Analysis when stored and used according to the manufacturer's instructions. No other warranty, expressed or implied (such as merchantability, fitness for a particular purpose, or non-infringement), is granted. Warranty is valid until the expiration date stated on the product label.

Warranty will be void if product is stored incorrectly, the recommended protocol is not followed, or the product is used for a different application.

5.3. Ordering Information / Support

When ordering with a Purchase Order:

E-mail a copy of the order to Cellc-sales@revvity.com

For online orders, please visit:

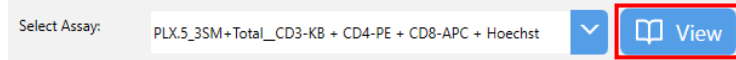
<https://www.revvity.com/cellcountingreagents>

For support, e-mail Cellc-support@revvity.com

6. Appendix

6.1. Assay Settings

6.1.1. To edit or review assay settings, click the **View** button next to the selected assay



6.1.2. Click the downward facing arrow in **Imaging and Analysis** to edit or review settings



NOTE: Below are the default assay settings for the Cellaca® PLX, anti-human CD3 KB520 / CD4 PE / CD8 APC Total Cell Kit

Imaging and Analysis

Imaging Mode

BR BR/FL Number of Channels: 4

Two-Color Fluorescence w/ Brightfield Imaging: Accurate Cell Counts / Viability / Dual FL Expression

Analysis Mode

Expression

Dual Fluorescence Analysis For Samples Containing Two FL Stains

Mask: BR FL

Use the channel selected as the FL mask channel

Expand (µm):

Amount, in microns, to expand or contract the found mask object which is used to collect FL intensity measurements in all channels

Focusing Mode

Focus Map Auto Focus 1st Well Auto Focus All Wells

Auto Focus Is Applied To Every Well For Best Focus/Image Quality

Auto Focus Image:

Dilution

Dilution Factor For General Assay As Indicated By Sample Preparation Protocol

NOTE: Below are the default Imaging Parameters for each channel in the Cellaca® PLX, anti-human CD3 KB520 / CD4 PE / CD8 APC Total Cell Kit

Channel 1 Channel 2 Channel 3 Channel 4

Imaging Parameters

Brightfield
 Use Custom Exposure:
 Custom Exposure Factor:

Fluorescence
 Fluorophore Name:
 Exposure (ms):

Filters
 Excitation: 365 470 531 620
 Emission: 452 534 605 655 692

Cell Type Parameters 3SM+Total_CD3-KB + CD4-PE + CD8-APC + Hoechst

Channel 1 Channel 2 Channel 3 Channel 4

Imaging Parameters

Brightfield
 Use Custom Exposure:
 Custom Exposure Factor:

Fluorescence
 Fluorophore Name:
 Exposure (ms):

Filters
 Excitation: 365 470 531 620
 Emission: 452 534 605 655 692

Channel 1 Channel 2 Channel 3 Channel 4

Imaging Parameters

Brightfield
 Use Custom Exposure:
 Custom Exposure Factor:

Fluorescence
 Fluorophore Name:
 Exposure (ms):

Filters
 Excitation: 365 470 531 620
 Emission: 452 534 605 655 692

Channel 1 Channel 2 Channel 3 Channel 4

Imaging Parameters

Brightfield
 Use Custom Exposure:
 Custom Exposure Factor:

Fluorescence
 Fluorophore Name:
 Exposure (ms):

Filters
 Excitation: 365 470 531 620
 Emission: 452 534 605 655 692

6.2. Cell Type Parameters

6.2.1 To edit or review assay settings, click the **View** button next to the selected assay

Select Assay: PLX.5_3SM+Total_CD3-KB + CD4-PE + CD8-APC + Hoechst

6.2.2 Click the downward facing arrow in **Imaging and Analysis** to edit or review settings

Imaging and Analysis

6.2.3 In **Imaging Parameters**, ensure Channel 1 is selected to view **Cell Type Parameters**

6.2.4 Ensure that the **Cell Type Parameter** selected corresponds to the kit being used

Cell Type Parameters 3SM+Total_CD3-KB + CD4-PE + CD8-APC + Hoechst

6.2.5 To edit or review Cell Type Parameters, click the **View** button

NOTE: Below are the default Cell Parameters for the Cellaca® PLX, anti-human CD3 KB520 / CD4 PE / CD8 APC Total Cell Kit

Brightfield Parameters

Cell Attributes	Decustering	Trypan Blue
Cell Diameter (µm): 2.0 to 22.0	Edge Factor: 0.7	Dead Cell Diameter (µm): 4.0 to 50.0
Roundness: 0.05	Threshold Factor: 1.0	Sensitivity: 1.0
Contrast Enhancement: 0.80	Background Adjustment: 1.0	Uniformity: 150
		Very Dim Dead Cells: No Yes
		Contrast Enhancement: 0.60

Fluorescence Parameters

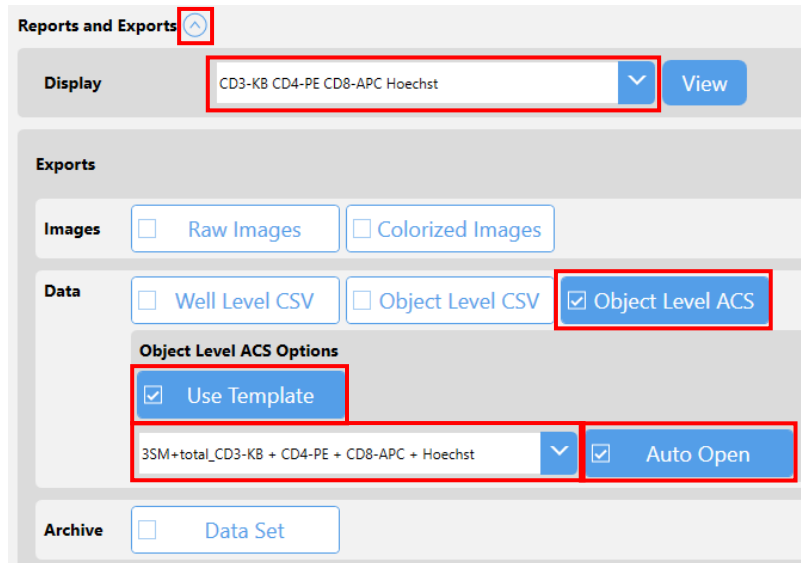
Cell Attributes	Thresholding
Cell Diameter (µm): 4.0 to 50.0	% of Image Range to Count: 10
Normalize intensity for cell size: No Yes	Threshold Factor: 1.0
Non-Uniform Cells: No Yes	
Roundness: 0.10	
Do Not Count Free Nuclei: No Yes	
Advanced BR/F Mode: No Yes	

6.3. Auto Export Data and Images

6.3.1 To edit or review assay settings, click the **View** button next to the selected assay



6.3.2 Click the downward facing arrow in **Reports and Exports** to edit or review settings



6.3.3 In **Display**, ensure the correct display is selected

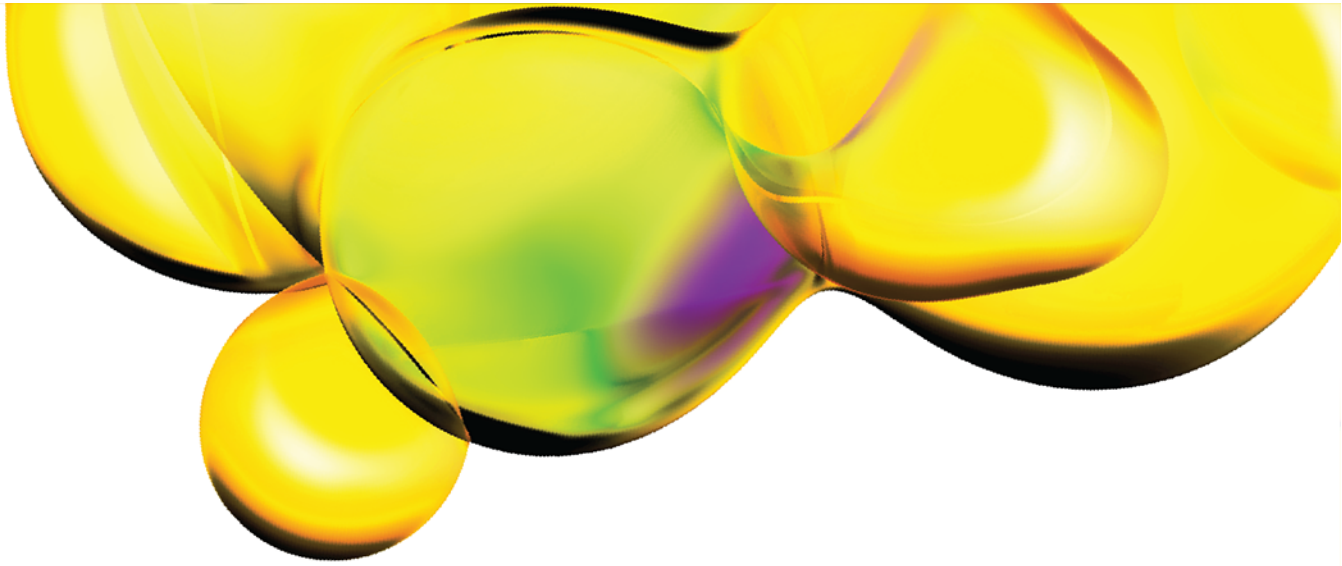
6.3.4 In **Exports**, select what you would like to be automatically exported after each scan when using this assay

6.3.4.1 For automatic export to FCS Express for surface marker analysis, select **Object Level ACS**, ensure **Use Template** is selected, and that the appropriate Template is selected, with the **Auto Open** button selected

6.4. Recommended Surface Marker and Total Dye Exposure Times and Filter Pairs

Recommended imaging parameters and exposure times (with ranges) for CD3, CD4, CD8 surface markers and Hoechst total dye on Cellaca® PLX Low Fluorescence slides. Exposure times may require optimization due to the individuality of each patient sample or cell line.

Cellaca® PLX Excitation / Emission	Illumination	Reagent	Assay Default Exposure Time (ms) (Recommended range)
365 / 452	Blue	Hoechst 33342	600 (400 – 800)
470 / 534	Green	CD3 KB520	1,000 (800 – 1,500)
531 / 605	Orange	CD4 PE	1,000 (800 – 1,500)
620 / 692	Far Red	CD8 APC	4,500 (3,000 – 6,000)



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