Cellaca® PLX, anti-human CD3 KB520 / CD4 PE Viability Kit
CSK-A0024-1 (25 Tests)
CSK-A0024-2 (100 Tests)
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1. Introduction

1.1. Description

CD3/CD4 surface marker reagents with viability dyes are designed for researchers interested in acquiring data on two surface marker populations and viability, as each patient and cell line derived sample can be unique. The Cellaca® PLX provides users with fluorescent and bright field images of their CD3/CD4 stained cells together with dead (RubyDead) and total (Hoechst) 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 populations with viability dyes on the Cellaca® PLX. The anti-human CD3 antibody is conjugated with KIRAVIA Blue 520™ and the anti-human CD4 antibody is conjugated with PE. For viability, dead cells are identified using the RubyDead dye, while total cells are stained with Hoechst. See table below for kit components and corresponding surface markers with their respective isotype controls.

<table>
<thead>
<tr>
<th>Cellaca® PLX Assay</th>
<th>Reagents</th>
<th>Catalog Number</th>
<th>Number of Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLX.5_2SM+Viab__</td>
<td>KIRAVIA Blue 520™ anti-CD3 (UCHT1) (Component A)</td>
<td>CSK-A0024-1</td>
<td>25</td>
</tr>
<tr>
<td>CD3-KB + CD4-PE +</td>
<td>PE anti-human CD4 (RPA-T4) (Component B)</td>
<td>CSK-A0024-2</td>
<td>100</td>
</tr>
<tr>
<td>Hoechst +</td>
<td>KIRAVIA Blue 520™ Mouse IgG1 Isotype (Component C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RubyDead</td>
<td>PE Mouse IgG1 Isotype (Component D)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hoechst 33342 (Component E)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RubyDead Dye (Component F)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.3. Required Materials

1.3.1. Cellaca® PLX instrument
1.3.2. Nexcelom-provided Laptop with Matrix 5.0 Software or above (pre-installed)
1.3.3. FCS Express software (pre-installed on Nexcelom-provided laptop)
1.3.4. Cellaca® PLX Low Fluorescence Slides (Cat. # CHM2-ACR)
1.3.5. Cellaca® PLX slide holder
1.3.6. Reagents provided in kit CSK-A0024
1.3.7. 1X Phosphate Buffered Saline
1.3.8. DMSO
1.3.9. Microcentrifuge tubes
1.3.10. Cell culture media
1.3.11. Cells or PBMC’s

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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 with Hoechst and RubyDead

<table>
<thead>
<tr>
<th>Cellaca® PLX Assay</th>
<th>Reagents</th>
<th>Catalog Number</th>
<th>Number of Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLX.5_2SM+Viab__</td>
<td>KIRAVIA Blue 520™ anti-CD3 (UCHT1) (Component A)</td>
<td>CSK-A0024-1</td>
<td>25</td>
</tr>
<tr>
<td>CD3-KB + CD4-PE +</td>
<td>PE anti-human CD4 (RPA-T4) (Component B)</td>
<td>CSK-A0024-2</td>
<td>100</td>
</tr>
<tr>
<td>Hoechst + RubyDead</td>
<td>KIRAVIA Blue 520™ Mouse IgG1 Isotype (Component C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PE Mouse IgG1 Isotype (Component D)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hoechst 33342 (Component E)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RubyDead Dye (Component F)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For each sample:

1. For a single sample, prepare 2 microcentrifuge tubes with 1 x 10⁶ PBMCs/cells each
   - **NOTE 1**: For 1 x 10⁶ cells, take 1 mL of 1 x 10⁶ cells/mL
   - **NOTE 2**: For multiple samples, prepare 2 tubes each

2. Label tubes, accordingly, one for staining with antibodies (Ab) and one for isotype control (Ctrl) staining for each distinct sample

3. Centrifuge cells at 1200 rpm for 5 minutes

4. Remove supernatant from all tubes avoiding cell pellets

5. Dilute Hoechst 33342 by adding 1 µL of Hoechst 33342 (Component E) to 19 µL 1X PBS
   - **NOTE**: 1:20 dilution for 1 mM working stock

6. Dilute RubyDead Dye by adding 1.5 µL of RubyDead Dye (Component F) to 1.5 µL DMSO
   - **NOTE 1**: 1:2 dilution for 100X working stock
   - **NOTE 2**: If staining 2-4 samples, prepare additional RubyDead Dye, according to the table below

<table>
<thead>
<tr>
<th></th>
<th>2 samples</th>
<th>3 samples</th>
<th>4 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>RubyDead Dye (Component F)</td>
<td>3 µL</td>
<td>4 µL</td>
<td>5 µL</td>
</tr>
<tr>
<td>DMSO</td>
<td>3 µL</td>
<td>4 µL</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

7. Resuspend the cell pellets from all tubes in 90 µL of cell culture media
   - **NOTE**: Staining with PBS results in dimmer signal
8. For staining cells in **Ab tubes**, add the following, and mix well:
   - 5 µL of **CD3 KB520** (Component A)
   - 5 µL of **CD4 PE** (Component B)
   - 1 µL of Hoechst working stock (diluted from step 5)
   - 1 µL of RubyDead Dye working stock (diluted from step 6)

   **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 11.5 µL of the master mix stain to each **Ab tube** and mix well.

<table>
<thead>
<tr>
<th></th>
<th>2 samples</th>
<th>3 samples</th>
<th>4 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 KB520 (Component A)</td>
<td>10 µL</td>
<td>15 µL</td>
<td>20 µL</td>
</tr>
<tr>
<td>CD4 PE (Component B)</td>
<td>10 µL</td>
<td>15 µL</td>
<td>20 µL</td>
</tr>
<tr>
<td>Hoechst working stock (Diluted from step 5)</td>
<td>2 µL</td>
<td>3 µL</td>
<td>4 µL</td>
</tr>
<tr>
<td>RubyDead Dye working stock (Diluted from step 6)</td>
<td>2 µL</td>
<td>3 µL</td>
<td>4 µL</td>
</tr>
</tbody>
</table>

9. For staining cells in **Ctrl tubes**, add the following, and mix well:
   - 5 µL of **IgG1 KB520** (Component C)
   - 2.5 µL of **IgG1 PE** (Component D)
   - 1 µL of Hoechst working stock (diluted from step 5)
   - 1 µL of RubyDead Dye working stock (diluted from step 6)

   **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 µL of the isotype control master mix stain to each **Ctrl tube** and mix well.

<table>
<thead>
<tr>
<th></th>
<th>2 samples</th>
<th>3 samples</th>
<th>4 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1 KB520 (Component C)</td>
<td>10 µL</td>
<td>15 µL</td>
<td>20 µL</td>
</tr>
<tr>
<td>IgG1 PE (Component D)</td>
<td>5 µL</td>
<td>7.5 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>Hoechst working stock (Diluted from step 5)</td>
<td>2 µL</td>
<td>3 µL</td>
<td>4 µL</td>
</tr>
<tr>
<td>RubyDead Dye working stock (Diluted from step 6)</td>
<td>2 µL</td>
<td>3 µL</td>
<td>4 µL</td>
</tr>
</tbody>
</table>

10. Incubate all tubes in the dark for 30 minutes at 4 °C

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

12. Centrifuge cells at 1200 rpm for 5 minutes

13. Remove supernatant from each tube avoiding cell pellets

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

   **NOTE:** Resuspension in 1X PBS results in dimmer signal

15. Mix samples thoroughly by pipetting up and down a few times
16. 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

17. Load 15 µL of sample from **Ctrl tube** into side B of the slide
   
   **NOTE**: Repeat for any additional samples prepared

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

19. 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

20. Proceed to section 4 for image and data acquisition
3. Expert User Quick Guide – CD3 KB520 / CD4 PE with Hoechst and RubyDead

Add 1 x 10^6 cells/tube

1200 rpm, 5 min

Remove supernatant
Resuspend cell pellet in 90 µL of media
Add reagents*

Incubate 4°C, 30 min

Add 200 µL 1X PBS

1200 rpm, 5 min

Resuspend with 100 µL media

Load samples into slides and image on Cellaca® PLX

* Dilute Hoechst 1:20 in 1X PBS
* Dilute RubyDead 1:2 in DMSO

<table>
<thead>
<tr>
<th>For Ab tubes:</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>CD3 KB520</td>
<td>5 µL 10 µL 15 µL 20 µL</td>
</tr>
<tr>
<td>CD4 PE</td>
<td>5 µL 10 µL 15 µL 20 µL</td>
</tr>
<tr>
<td>Hoechst</td>
<td>1 µL 2 µL 3 µL 4 µL</td>
</tr>
<tr>
<td>RubyDead</td>
<td>1 µL 2 µL 3 µL 4 µL</td>
</tr>
</tbody>
</table>

Add 11.5 µL of the master mix to each tube

<table>
<thead>
<tr>
<th>For Ctrl tubes:</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>IgG1 KB520</td>
<td>5 µL 10 µL 15 µL 20 µL</td>
</tr>
<tr>
<td>IgG1 PE</td>
<td>2.5 µL 5 µL 7.5 µL 10 µL</td>
</tr>
<tr>
<td>Hoechst</td>
<td>1 µL 2 µL 3 µL 4 µL</td>
</tr>
<tr>
<td>RubyDead</td>
<td>1 µL 2 µL 3 µL 4 µL</td>
</tr>
</tbody>
</table>

Add 9 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 the **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 and Dead 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.

<table>
<thead>
<tr>
<th>Iteration</th>
<th>Tube</th>
<th>File Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample 1</td>
<td>object_1.acs</td>
</tr>
<tr>
<td>2</td>
<td>Sample 1 (isotype)</td>
<td>object_1.acs</td>
</tr>
<tr>
<td>3</td>
<td>Sample 2</td>
<td>object_2.acs</td>
</tr>
<tr>
<td>4</td>
<td>Sample 2 (isotype)</td>
<td>object_2.acs</td>
</tr>
</tbody>
</table>
5. Additional Resources

5.1. Storage and Handling
Store each product at 4 °C, protected from light. Please consult the Safety Data Sheet for more safety information, found on www.nexcelom.com/Products.

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:
   - Fax a copy of the order to 978-327-5341
   - Email a copy of the order to Cellc-sales@perkinelmer.com
   - Email support at Cellc-support@perkinelmer.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 Viability Kit.
NOTE: Below are the default Imaging Parameters for each channel in the Cellaca® PLX, anti-human CD3 KB520 / CD4 PE Viability Kit

6.2. Cell Type Parameters

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

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

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.

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 Viability Kit.
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 and Dead Dye Exposure Times and Filter Pairs

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

<table>
<thead>
<tr>
<th>Cellaca® PLX Excitation / Emission</th>
<th>Illumination</th>
<th>Reagent</th>
<th>Assay Default Exposure Time (ms) (Recommended range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>365 / 452</td>
<td>Blue</td>
<td>Hoechst 33342</td>
<td>600 (400 – 800)</td>
</tr>
<tr>
<td>470 / 534</td>
<td>Green</td>
<td>CD3 KB520</td>
<td>1,000 (800 – 1,500)</td>
</tr>
<tr>
<td>531 / 605</td>
<td>Orange</td>
<td>CD4 PE</td>
<td>1,000 (800 – 1,500)</td>
</tr>
<tr>
<td>620 / 692</td>
<td>Far Red</td>
<td>RubyDead</td>
<td>1,200 (1,000 – 1,500)</td>
</tr>
</tbody>
</table>