Life Science Research Application Note

Guideline for the Use and Optimization of User Defined Markers: CellSearch® Epithelial Cell Kit and CellSearch® CXC Kit

The CellSearch® Epithelial Cell Kit and the CellSearch® CXC Kit were designed to provide researchers with the capability to investigate the presence of biomarkers associated with circulating epithelial cells. This enables researchers to further characterize circulating epithelial cells with a marker of their choice. This guideline provides researchers with directions for use and optimization of user-defined markers with the CellSearch® Epithelial Cell Kit and the CellSearch® CXC kit.

The CellSearch® Epithelial Cell Kit and the CellSearch® CXC Kit are for Research Use Only and are not for use in diagnostic procedures. The performance characteristics and safety and effectiveness have not been established and are not cleared or approved by the FDA. The methods contained within this document may not be optimal for all research applications. Researchers must confirm the utility of these methods for their application through the use of well designed and appropriately controlled experiments.

Introduction

The CellSearch® Epithelial Cell Kit and the CellSearch® CXC Kit contain a ferrofluid-based capture reagent and immunofluorescent staining reagents to capture and detect circulating epithelial cells. The ferrofluid consists of nanoparticles with a magnetic core surrounded by a polymeric layer coated with antibodies targeting the Epithelial Cell
Adhesion Molecule (EpCAM) antigen for capturing circulating epithelial cells. After immunomagnetic capture and enrichment, fluorescent staining reagents are added for identification and enumeration of these cells.

The CellSearch® Epithelial Cell Kit uses anti-CK-Phycoerythrin (PE) to label the intracellular protein cytokeratin (specific for epithelial cells), 4'-6-Diamidino-2-phenylindole (DAPI) to label the cell nucleus, and anti-CD45-Allophycocyanin (APC) to label leukocytes. The CellSearch® CXC Kit uses the same reagents for the nucleus and CD45 (leukocytes), but uses anti-CK-Fluorescein Isothiocyanate (FITC) for labeling instead of PE. There is one additional channel on the CellTracks Analyzer II® instrument (CTAII) that can be used for a user-defined marker. The CellSearch® Epithelial Cell Kit leaves the dihexyloxacarboeyanine iodide (DIOC)/FITC channel open and the CellSearch® CXC Cell Kit leaves the PE channel open.

The open channel enables researchers to process and analyze samples with one additional user-defined marker. This enables researchers to further characterize circulating epithelial cells with a marker of their choice that has been conjugated to an appropriate fluorochrome.

The CellSearch® CXC kit was developed to allow identification of tumor markers on circulating epithelial cells of moderate to low antigen density. The CellSearch® Epithelial Cell Kit used in conjunction with a user-defined marker reagent allows identification of tumor markers on circulating epithelial cells of high antigen density.

**Table 1:**
**User-Defined Markers: CellSearch® Epithelial Cell Kit vs. CellSearch® CXC Kit**

<table>
<thead>
<tr>
<th></th>
<th>CellSearch® Epithelial Cell Kit</th>
<th>CellSearch® CXC Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity</strong></td>
<td>Markers on Circulating Epithelial Cells with higher antigen density (~100,000 antigens/cell or greater)</td>
<td>Markers on Circulating Epithelial Cells with moderate to low antigen density (~50,000 antigens/cell or greater)</td>
</tr>
<tr>
<td><strong>Fluorochrome</strong></td>
<td>Fluorescein (FITC)</td>
<td>PE (Phycoerythrin)</td>
</tr>
<tr>
<td><strong>Conjugate for Marker Reagent</strong></td>
<td><strong>Veridex optimized marker reagents</strong></td>
<td><strong>NO</strong></td>
</tr>
<tr>
<td></td>
<td>YES HER-2/neu (P/N 7900006)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EGFr (P/N 7900011)</td>
<td></td>
</tr>
<tr>
<td><strong>Compatible Operating System(s)</strong></td>
<td>CellTracks Analyzer II® – XP</td>
<td>CellTracks Analyzer II® – Linux</td>
</tr>
<tr>
<td></td>
<td>CellTracks Analyzer II® - Linux</td>
<td></td>
</tr>
<tr>
<td><strong>Control Cells Available?</strong></td>
<td><strong>YES</strong> (P/N 7900002)</td>
<td><strong>YES</strong> (P/N 7900018)</td>
</tr>
</tbody>
</table>

* IGF-1r-PE is a marker reagent that is available from a commercial vendor and has been optimized for use with the CellSearch® CXC Kit. Please refer to the FAQ section for recommended parameters.
Guideline for Optimization of User-Defined Markers

It is important to optimize the parameters for user-defined markers because failure to do so may lead to misinterpretation of results or inaccurate conclusions.

Optimization Parameters

There are two parameters that are available for optimization: Antibody Concentration and Exposure/Integration Time.

Antibody Concentration:

The volume of antibody that is added to the sample during processing on the CellTracks® AutoPrep® is fixed (150 µL antibody solution in 850 µL reaction volume). Therefore, it is essential that the concentration of the conjugated antibody for the marker of interest be optimized to work under these conditions. The optimal concentration will depend on many factors such as antigen density, location, epitope availability and antibody specificity and affinity.

Examples of optimized antibody stock concentrations: Her-2/neu-FITC and EGFr-FITC (6 µg/mL), IGF-1r-PE (see FAQ section)

Stock concentration: the concentration of the marker reagent in the cup
Working concentration: the final concentration of the marker reagent once added to the reaction mixture.

Note: It is recommended that marker reagent be diluted using 1X PBS (at physiologic pH) with or without protein (e.g. 0.1% BSA) and that the diluted marker reagent be filtered using a 0.4µm, pre-wetted, syringe-tip, low protein-binding filter (e.g. polyethersulfone).

Exposure/Integration Time:

Exposure/Integration time is a parameter associated with the CellTracks Analyzer II® camera settings. The setting represents the amount of time that the shutter of the camera is open to allow light to pass through at a given wavelength. The longer the integration time the more signal will be collected. Therefore, if the signal from a conjugated marker is very dim, the exposure time may need to be longer. However if the integration time is set too long, background signal will increase leading to non-specific signal or artifact. Conversely, if the exposure time is set too short, then a positive signal may not be detected.

The exposure time should be kept as low as possible while still maintaining sufficient positive signal over background.

Refer to Appendix 2 for setup of the CellTracks® System and for antibody volume and cup to use with the AutoPrep® System.
Optimization Controls

The marker reagent should be optimized to demonstrate that the reagent can accurately identify the marker of interest on circulating epithelial cells within the CellSearch® System.

This requires having predictable controls when optimizing.

- **Known positive High Antigen Density cell line** – an EpCAM+/CK+ tumor cell line that is known to express high levels of the marker of interest. This will help to ensure the antibody being used is specific for the marker of interest.
- **Known positive Low Antigen Density cell line** – an EpCAM+/CK+ tumor cell line that is known to express low levels of the marker of interest. This will help to ensure the antibody being used is sensitive enough for the marker of interest.
- **Known negative cell line** – an EpCAM+/CK+ tumor cell line that does not express the marker of interest. This will help to ensure the antibody being used is specific for the marker of interest.

The cell lines should be spiked into normal blood samples and processed with and without the user-defined marker added. Processing a positive control cell line in the absence of marker under the same analysis parameters used for the marker, will help demonstrate any background signal that may be associated with the exposure time being used to analyze samples.

Refer to Appendix 1 for an example of parameter optimization
FAQ’s for User-Defined Markers

1. How much of a given antigen must be present on my cells of interest to be detected?
   Answer: Antigen detection can depend on distribution, type and location of the marker of interest. Refer to Table 1 for suggestions.

2. What is the correct concentration for my antibody?
   Answer: Refer to the Optimization Parameters section and Appendix 1

3. What dilution buffer should I use for my antibody?
   Answer: 1X PBS, with or without protein (0.1% protein) at physiologic pH (e.g. pH 7.4)

4. Do I need to filter my marker reagent before using it with the system?
   Answer: Yes, it is recommended that the diluted marker reagent be filtered using a 0.4 µm, pre-wetted, syringe-tip, low protein-binding filter (e.g. polyethersulfone).

5. Are there marker reagents that are available from commercial vendors other than Veridex that may be used that have parameter recommendations?
   Answer: Yes, IGF-1r is a marker reagent that may be used with the CellSearch® CXC Kit. Anti-IGF-1r-PE, clone 1H7, is available from BD Pharmingen. The recommended stock concentration can be prepared by performing a 1:3 dilution with 1X PBS, followed by filtration. The exposure time that is recommended is between 0.4 and 0.6 (s). Note: These conditions may need to be adjusted depending on the system or original concentration of the antibody solution from the vendor.

6. How do I adjust exposure time?
   Answer: Refer to Appendix 2

7. What are the optical specifications for the filter cubes used on the CTAII?
   Answer: This information can be found in the CTAII User’s Guide in the Specifications section

8. Can other fluorochrome conjugates be used besides FITC for the CellSearch® Epithelial Cell Kit and PE for the CellSearch® CXC Kit?
   Answer: It may be possible to use other fluorochromes, however no information is available to suggest use of fluorochromes other than FITC (FLUOR) for the CellSearch® Epithelial Cell Kit and PE for the CellSearch® CXC Kit.

9. How much marker reagent volume do I need to use to run samples?
   Answer: There is a required excess volume of 300 µL. This means that an additional 300 µL of marker reagent needs to be added for each batch.
Add diluted marker reagent to the reagent cup according to the number of samples that will be processed with the marker. The table below provides a guideline for the volume requirement based on batch size.

<table>
<thead>
<tr>
<th># Samples in batch to be processed with marker reagent</th>
<th>Reagent Volume (µL) to be added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>450</td>
</tr>
<tr>
<td>2</td>
<td>600</td>
</tr>
<tr>
<td>3</td>
<td>750</td>
</tr>
<tr>
<td>4</td>
<td>900</td>
</tr>
<tr>
<td>5</td>
<td>1050</td>
</tr>
<tr>
<td>6</td>
<td>1200</td>
</tr>
<tr>
<td>7</td>
<td>1350</td>
</tr>
<tr>
<td>8</td>
<td>1500</td>
</tr>
</tbody>
</table>

Do not add more volume than what is specified in the table above.

10. What kind of tube/cup do I use on the system for my marker reagent?

   **Answer**: Only use the cups provided with CellSearch® CXC kit or TPR kits. If neither the CellSearch® CXC kit or a TPR kit is being used, then the following cup must be used:

<table>
<thead>
<tr>
<th>Manufacturer Name</th>
<th>Simport Plastics LTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part Number</td>
<td>T341-6T</td>
</tr>
<tr>
<td>Description</td>
<td>Clear, 2.0mL tube</td>
</tr>
<tr>
<td>Supplier Name</td>
<td>Biomedical Associates*</td>
</tr>
</tbody>
</table>

   *also available from through other vendors (e.g. VWR)

11. What exposure time should I use?

   **Answer**: Refer to the Optimization Parameters section

12. Can I change the exposure time of other filters besides the marker filter?

   **Answer**: No – it is not possible to edit the exposure times of the other filters on the Linux platform. The exposure times have been optimized for the other channels and must not be altered on the XP platform either. Altering these channels may produce erroneous results.

13. Is it possible to use more than one marker reagent?

   **Answer**: No, only one marker reagent can be used at a time

14. Can I use a primary antibody followed by a secondary antibody to amplify signal?

   **Answer**: No, marker reagents must be directly conjugated.

15. What are the antibody isotypes used for the staining reagent in the CellSearch® kits?

   **Answer**: Murine monoclonal IgG1 or IgG2a
16. Can I use polyclonal antibodies?
   **Answer:** Yes, for best results, the antibody should be affinity purified and filtered.

17. Can I re-use the leftover volume/dead volume?
   **Answer:** No, it is not recommended, as the left over marker reagent may no longer be pure.

18. Can I customize the volume of marker reagent added by the system or the incubation times?
   **Answer:** No, it is not possible to alter these parameters.

19. Can I label my own antibody?
   **Answer:** Yes. The recommended Antibody:Fluorochrome ratios are:
   - 1:5 - 1:10 (FITC)
   - 1:1 (PE)

20. Will the exposure parameter setting be the same on different instruments?
   **Answer:** The exposure parameter should be confirmed and may need slight adjustment between one instrument and another instrument.

21. Will CTC recovery be the same when using the CellSearch® CXC kit as compared to the CellSearch® Epithelial Cell Kit?
   **Answer:** The data on file for the CellSearch® CXC kit is similar to the data on file for the CellSearch® Epithelial Cell kit with regard to the proportion of samples that are circulating epithelial cell positive. However, the number of Circulating Epithelial Cells detected with CellSearch® CXC kit may be 10 to 18% lower when compared to the CellSearch® Epithelial Cell kit. The lower recovery may be due to some Circulating Epithelial Cells which have lower cytokeratin expression level that are not being detected with the CellSearch® CXC kit.
   The CellSearch® CXC kit was developed for the identification of tumor markers on Circulating Epithelial Cells of moderate to low antigen density. The lower recovery does not impact this identification. The information derived from the use of the CellSearch® CXC kit should be based on the specific tumor marker expression on identified circulating epithelial cells, and not on absolute number.
Appendix 1

Example of Optimization of User-Defined Marker Reagent using the CellSearch® CXC Kit

Selection of Optimization Controls

Figure A. Flow cytometry analysis was used to characterize three cell lines with different antigen densities to be used as controls for optimization. Antibody titrations were run with each cell line to determine optimal concentration. Only the optimal concentration and exposure time is shown in this figure. MCF-7: Low Density, A-431: Medium Density, SNU-5: High Density. SKBR3 cells (not shown) are known not to express the antigen of interest and can be used as a negative control cell.

Figure B, C and D. CellTracks® analysis of the three cell lines where the marker expression relative to one another is low (MCF-7), medium (A-431) and high (SNU-5).
Establishment of Background

A. Negative Cell Line SKBR-3
B. Positive (Low) Cell Line MCF-7
C. Positive (Medium) Cell Line A-431

Three different cell lines (negative, positive-low antigen density and positive-high antigen density) processed in the absence of marker reagent to establish background signal at a given exposure time. In this example, any signal observed in the PE channel is classified as negative.
Establishment of Background - Continued

The low antigen density cell line (MCF-7) processed in the absence of marker reagent to establish background signal at a four different PE exposure times: 0.2, 0.3, 0.4, and 0.5 seconds.

The data shows a faint image in the PE channel which is mostly due to reflected light in the channel and autofluorescence associated with cultured cells. The image gets more pronounced as the exposure time increases to 0.4 and 0.5 seconds.

The images seen at exposure times of 0.2 and 0.3 seconds are representative of the typical background seen with cultured cells.

These background images will be used later to establish positive signal verses background signal. A positive signal should be strong enough such that it is easily discernable from background signal.
Titration of User-Defined Marker Antibody – Medium Antigen Density Cell Line

Positive - Medium Antigen Density Marker Cell Line (A-431)

A. 0.10 µg/mL Marker PE  
B. 0.15 µg/mL Marker PE

C. 0.20 µg/mL Marker PE  
D. 0.25 µg/mL Marker PE

Titration of the Marker-PE antibody to determine minimum antibody concentration for the medium-density cell line to achieve a positive signal – minimum concentration: 0.15 µg/mL.

Concentration is expressed as the working concentration which is the final concentration of the marker reagent once added to the reaction mixture. Stock concentration, which is the concentration of the antibody in the cup prior to addition to the sample, can be calculated by:

\[ \text{[working concentration in } \mu\text{g/mL}] \times [850 \mu\text{L}] / 150 \mu\text{L} = \text{stock concentration in } \mu\text{g/mL}. \]
**Titration of User-Defined Marker Antibody – Low Antigen Density Cell Line**

Higher marker-PE antibody concentrations tested with low-density cell line – to determine the lowest antibody concentration that can be used with the low-density cell line to achieve a positive signal – minimum concentration: 2.0 µg/mL. Exposure Time: 0.5 s

Concentration is expressed as the working concentration which is the final concentration of the marker reagent once added to the reaction mixture. Stock concentration, which is the concentration of the antibody in the cup prior to addition to the sample, can be calculated by:

\[
\text{stock concentration in } \mu\text{g/mL} = \frac{\text{working concentration in } \mu\text{g/mL} \times 850 \mu\text{L}}{150 \mu\text{L}}
\]

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Optimization of Exposure Time – Low Antigen Density Cell Line

Figure A

Exposure Time of 0.2 Seconds

Exposure Time of 0.3 Seconds

Exposure Time of 0.4 Seconds

Exposure Time of 0.5 Seconds

Figure B

Exposure Time of 0.2 Seconds

Exposure Time of 0.3 Seconds

Exposure Time of 0.4 Seconds

Exposure Time of 0.5 Seconds

Figure A: The cell line MCF-7 processed with marker PE at 2 µg/mL with PE exposure times of 0.2, 0.3, 0.4, and 0.5 seconds. The data shows insufficient signal relative to background at all integration times tested at this concentration of marker PE. Marker concentration should be increased.

Figure B: The cell line MCF-7 processed with marker PE at 4 µg/mL with PE exposure times of 0.2, 0.3, 0.4, and 0.5 seconds. The data shows sufficient signal relative to background beginning at an integration time of 0.3 seconds at this marker PE concentration.
Titration of User-Defined Marker Antibody – Negative Cell Line

Negative Marker Cell Line (SKBR)

A. 0.5 µg/mL Marker PE
B. 1.0 µg/mL Marker PE
C. 2.0 µg/mL Marker PE
D. 4.0 µg/mL Marker PE

Higher marker-PE antibody concentrations tested were with negative cell line to determine the highest antibody concentration that can be used with the negative cell line to achieve a negative signal as compared to signal observed with positive cell lines— all results were negative for marker.

Concentration is expressed as the working concentration which is the final concentration of the marker reagent once added to the reaction mixture. Stock concentration, which is the concentration of the antibody in the cup prior to addition to the sample, can be calculated by:

\[
\text{[working concentration in µg/mL]} \times [850 \text{ µL}] / 150 \text{ µL} = \text{stock concentration in µg/mL.}
\]
Summary of Results

- The optimal working parameters for the marker reagent in this system were determined to be 4.0 µg/mL with an exposure time of 0.3 seconds.

Notes: The optimal parameters listed above are specific to this example and should not be considered optimal for all markers – each marker must be optimized individually for concentration and exposure time. The optimal concentration can vary widely, depending on the marker of interest. Typically, the optimal exposure time for the DIOC filter does not exceed 0.8 seconds and for the PE filter does not exceed 0.6 seconds.

- The marker reagent specificity was verified by using a cell lines with low, moderate and high antigen densities and then titrating with low and moderate antigen density cell lines.

- The exposure time was verified by running the positive control cell line with and without marker reagent added.

- The optimal concentration was selected based on the minimum marker reagent concentration required to obtain a positive result when using the cell line with low antigen density.

- A positive result was defined by having a clear image in the marker reagent channel, with a dark background (black or nearly black) and a clear difference between the positive signal achieved with the low antigen density cell line and the negative signal achieved with the negative cell line or the signal achieved when no marker reagent was added.
Appendix 2

CellTracks® System Setup for User Defined Markers

Creating a Name for a User-defined Assay on the CellTracks® AutoPrep® Setup Guideline

Use this procedure to process a sample with a marker reagent.

- Level One access is required for this function.

1. At the Main Menu, select Run Batch and enter your password.
2. Select the kit from the list (for example, CellSearch® Epithelial Cell Kit or the CellSearch® CXC Kit).
3. Select the protocol from the list
   - To use a default test protocol (for example, Her-2/neu), select the test and then click Next.
   - To set up a user-defined marker protocol:
     a. Select User Defined Assay and then click Next.
     b. Enter the Marker Name.

Note: The Marker name entered here must match the Marker ID entered on the CellTracks Analyzer II® (Linux). This Marker ID will appear on the Review gallery and on the CellTracks® Report.

4. Continue setting up the batch as usual.
5. Add the marker reagent to the cup provided with the kit according to the table below:

<table>
<thead>
<tr>
<th># Samples in batch to be processed with marker reagent</th>
<th>Reagent Volume (µL) to be added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>450</td>
</tr>
<tr>
<td>2</td>
<td>600</td>
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<tr>
<td>6</td>
<td>1200</td>
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<tr>
<td>7</td>
<td>1350</td>
</tr>
<tr>
<td>8</td>
<td>1500</td>
</tr>
</tbody>
</table>

Do not add more volume than what is specified in the table above.

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**Note:** Only use the cups provided with CellSearch® CXC kit or TPR kits. If neither the CellSearch® CXC kit or a TPR kit is being used, then the following cup must be used:

<table>
<thead>
<tr>
<th>Manufacturer Name</th>
<th>Simport Plastics LTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part Number</td>
<td>T341-6T</td>
</tr>
<tr>
<td>Description</td>
<td>Clear, 2.0mL tube</td>
</tr>
<tr>
<td>Supplier Name</td>
<td>Biomedical Associates*</td>
</tr>
</tbody>
</table>

*also available through other vendors (e.g. VWR)*

6. When prompted, load the Marker cup in position 1 of the reagent carrier.
   **Note:** ensure that the cup is fully inserted into the carousel so that the bottom sits flush. The cup should not have to be “forced” into position. If the fit seems too tight, then use another cup.

7. Load the reagent kit, MagNest® components, and samples onto the CellTracks® AutoPrep® System as usual.

8. When prompted, enter Yes or No to “Run Marker test on this sample?” for each sample.

9. Confirm your sample information and then press **Enter** to start the run.

**Defining Test Protocols for the CellTracks Analyzer II® – Linux**

User-defined test protocols can be created or existing protocols can be modified.

**Note:** Level 3 access is required for this function

*To modify the exposure time for an existing test protocol:*  

1. Click the **Utilities** tab.  
2. In the Test Protocols section, click the down arrow to select a kit from the Kit ID list.  
3. Select the protocol and then click **Edit**.

**Important:**

Exposure is the length of time (in seconds) that the camera shutter remains open while imaging the object. The default exposure times are not necessarily optimized for your system in your facility. Exposure times for all Marker protocols should be optimized for your research needs.
4. Enter the desired exposure time and then click **Save**.

*To create a user-defined test protocol:*

1. Click the **Utilities** tab.

2. In the Test Protocols section, select the appropriate kit from the Kit ID list. The standard marker protocols and a default research protocol are listed.

3. To add a new user-defined marker protocol, select a blank line and then click **New**.

**Important:**

- The Marker ID entered here must match the marker ID for this test on the CellTracks® AutoPrep® System. This Marker ID will appear on the Review gallery and on the CellTracks® Report.

- Exposure is the length of time (in seconds) that the camera shutter remains open while imaging the object. The default exposure times are not necessarily optimized for your system in your facility. Exposure times for all Marker protocols should be optimized for your research needs.

4. Enter the parameters as needed and then click **Save**.

**Note:** For the CellSearch® Epithelial Cell Kit and CellSearch® Endothelial Cell Kit protocols, the marker stain must be FITC-labeled. For the CellSearch® CXC Kit the marker stain must be PE-labeled.
Defining Test Protocols and Scanning with CellTracks Analyzer II® – XP

1. Click on the **Image Acquisition** button on the **Home Screen**.
2. Enter **User Name** and **Password**.
3. Click **OK** in the dialog box.
4. Choose **Research** as the test type
   
   Note: The XP Platform is not designed to process the CellSearch® CXC kit

Test selection loads test parameters within the software. The background color of the screen is blue for Research mode. Perform System Verification if you are scanning the first research sample of the day.

5. Adjust integration times according to package insert provided with the reagent.
   Access the configuration screen by clicking on the Advanced menu at the top of the Data Acquisition screen, and choose **Configure**.
   
   **Caution**: Do not change any fields except for filter name (up to 7 characters, do not use “/” in the name) and associated integration time for the FITC channel (Filter 2). The default integration time for FITC is set for a conjugate with moderate fluorescence intensity.
   The researcher is responsible for optimizing settings. **DO NOT** change the setting for any other filter.

6. Click **OK** to continue.

7. Set changes as new default or for current test.
8. Click OK to set the change filter names/integration times to new defaults. Click cancel to use the settings only for the current test.

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