Optimizing performance of Transcreener fluorescence polarization assays with the SpectraMax i3x Multi-Mode Microplate Reader

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Introduction

This application note describes the optimal instrument parameters used to validate the SpectraMax® i3x Multi-Mode Microplate Reader with the Transcreener® ADP² FP assay from BellBrook Labs.

The Transcreener FP Assays are a single step, competitive immunoassay for direct detection of nucleotides with a far-red fluorescence polarization (FP) readout. Reagents for all the assays include a far-red Tracer bound to a highly specific monoclonal/polyclonal antibody. The enzymatic reaction produces nucleotide diphosphate or monophosphate which displaces the tracer from the antibody. This results in the generation of a signal due to an increase in rotational freedom of the tracer, detected as a decrease in polarization, as depicted by Figure 1. The use of a far-red tracer minimizes interference from fluorescent compounds and light scattering. The Transcreener FP Assays are designed specifically for high-throughput screening (HTS) with a single addition, mix-and-read format.

Specifically, the Transcreener ADP² Assay with FP readout uses an antibody selective to ADP over ATP and a far-red fluorescent tracer. The ADP produced in the assay reaction competes with the tracer-labeled ADP for binding to the antibody. Consequently, fluorescence polarization will decrease with an increase in ADP production. This universal detection method allows screening of virtually any ADP-producing enzyme in a high throughput format.

The SpectraMax i3x reader offers a modular design allowing the addition of FP detection to the instrument in just minutes. Background noise is reduced by the reader’s cooled PMT, enabling increased sensitivity and dynamic range. Using optimized settings for the Transcreener FP assays presented in this application note, the SpectraMax i3x reader exceeds BellBrook Labs validation requirements.

Benefits

- Mix-and-read format gives robust results for high-throughput screening
- Far-read readout minimizes compound interference
- Z° values > 0.7 and Δ mP > 120 achieved with a read time under 3 minutes
Validation criteria

A critical factor in realizing the advantages of the Transcreener HTS assays is the correct setup of the microplate reader used for data readout. Proper selection of instrument settings can impact the instrument’s sensitivity for any given assay. The key instrument parameters for Transcreener HTS assay performance were identified by running a 10 μM ATP/ADP standard curve (24 replicates), as standard curves of this type mimic enzyme reactions. Starting with 10 μM ATP, ADP was added in increasing amounts and ATP was decreased proportionately, maintaining a total adenine nucleotide concentration of 10 μM. The integration times were varied to determine the optimal requirements for instrument validation. In order to validate an instrument for use with the Transcreener FP Assays a Z’ factor > 0.7 and a Δ mP > 120 at 10% conversion of 10 μM ATP were required.

Materials

- ATP/ADP Mixture in buffer solution (Buffer: 4 mM MgCl2, 2 mM EGTA, 50 mM HEPES, pH 7.5, 1% DMSO, 0.01% Brij-35). ATP/ADP are combined to a constant adenine concentration of 10 μM.
- ADP Detection Mixture: 1X Stop & Detect Buffer B, 4 nM ADP Alexa633 Tracer, and 14.8 μg/mL ADP2 Antibody
- Free Tracer: 1X Stop & Detect Buffer B and 4 nM ADP Alexa633 Tracer
- Buffer Blank: 1X Stop & Detect Buffer B and 14.8 μg/mL ADP2 Antibody


Methods

Assay preparation

1. Dispense 10 μL of each ATP/ADP combination across an entire row of a 384-well plate (rows A-O, see plate diagram).
2. Add 10 μL of ADP Detection Mix to those rows.
3. Dispense 10 μL of the 10 μM ATP/0 μM ADP combination into row P.
4. Dispense 10 μL of Free Tracer into wells P1-P12.
5. Dispense 10 μL of Buffer Blank into wells P13-P24.

\[
\text{Substrate} + \text{ATP} \xrightarrow{\text{Enzyme}} \text{ADP} + \text{Substrate}
\]

\[
\text{Fluorescence Polarization: ADP} \xrightarrow{\text{Y}} \text{ADP} \xrightarrow{\text{Y}} \text{ADP} - \text{ADP}
\]

Figure 1. Illustration of Transcreener ADP2 FP enzymatic reaction (BellBrook Labs). Increasing enzymatic activity leads to conversion of ATP to ADP, which competes with tracer for binding to the antibody. The more unbound tracer there is, the lower the fluorescence polarization.
Setup for detection of the Transcreener assay on a SpectraMax i3x reader is shown in Table 1.

The SpectraMax i3x reader is operated using SoftMax® Pro Software. Start with a pre-configured FP protocol and modify as described below. Make sure that the custom FP cartridge is installed before proceeding.


2. Click ‘Plate1’ in the Navigation Tree and click on Settings (gear icon) to open the Settings dialog.

3. Select the custom cartridge for Alexa Fluor 633 FP from the Cartridges list of the Optical Configuration settings.

4. Read Mode ‘FP’ and Read Type ‘Endpoint’ should be highlighted.

5. Wavelength settings are determined by the cartridge selected and are not user changeable.

6. Click ‘Plate Type’ and choose Plate Format ‘384 Wells’ and Select Specific ‘384 Well Corning low vol/rndbtm’ (or plate type used).

7. Click ‘Read Area’ and choose the wells to read on the plate diagram.


9. If Stop and Go is selected, enter an integration time of 30 ms or higher. Longer integration times typically offer better performance, but plate reading will take longer.

10. If optimal read height is known, enter it. For any new assay or plate type, read height optimization should be performed. If optimization will be performed, leave the read height at the 1 mm default value. After optimization is performed, the new (optimal) read height will replace the default value.

11. Click ‘More Settings’. Choose a read order, ‘Row’ if a whole plate or a few complete rows will be read, or ‘Column’ if only a few complete columns will be read.

12. Check the box next to ‘Show Pre-Read Optimization Options’.

13. Click ‘OK’ to close the Settings dialog.

14. Click Read. A wizard showing optimization options will appear. Perform both microplate optimization and read height optimization for any new assay or lot of plates.

Table 1. Recommended SpectraMax i3x reader settings. Pre-read optimization of the microplate and read height should be performed for each new plate type used.
Results

Sample FP standard curve

As the ratio of ADP to ATP increases, the proportion of bound tracer vs. free tracer decreases, resulting in an overall decrease in mP values. Assay plates containing the 15-point standard curve were read on a SpectraMax i3x reader.

Plots of the assay results vs. assay validation parameters are shown in Figure 2. Table 2 summarizes the results obtained with different integration times; assay performance requirements are exceeded using an integration time of only 30 ms, or the Performance on-the-fly (OTF) setting.

Figure 2. Assay validation parameters. A: Z’ and Δ mP values observed in a standard curve mimic conversion of 10 μM ATP to ADP. B: Zoomed view of the 0-2 μM ADP section of the standard curve shows the Z’ validation minimal qualification data (red dotted line) and Δ mP validation minimal qualification data (black dashed line). The 10% ATP conversion validation point is also indicated (vertical black dotted line).

<table>
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<th>Integration time</th>
<th>20 ms</th>
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<th>50 ms</th>
<th>100 ms</th>
<th>150 ms</th>
<th>200 ms</th>
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<td>133.1</td>
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</table>

Table 2. Assay performance with various instrument settings. Validation requirements are met using either an integration time of 30 ms, or On the Fly (Performance) setting.

Conclusion

This application protocol demonstrates the validation of the SpectraMax i3x reader for use with the Transcreener FP Assays. By utilizing the optimized instrument settings suggested here, Z’ values > 0.7 and Δ mP > 120 are achievable with read times of less than 3 minutes. Results greatly exceeding minimum validation requirements can be achieved by using longer integration times, while the faster on-the-fly setting meets assay requirements with a read time of less than two minutes.