**A Fluorescence-Based Neurotransmitter Uptake Assay**

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**Abstract**
Here we present a homogeneous assay of norepinephrine, dopamine, and serotonin transporter activity based on cell uptake of a fluorescent dye coupled with a proprietary masking dye. In this no-wash kit recently introduced by Molecular Devices, cells expressing the transporter of interest are incubated with the dye/reagent mix and transferred to a plate reader for evaluation. The assay can be performed in 96- or 384-well microtiter plates and read on any fluorescence microplate reader in bottom-read mode. Either an endpoint or kinetic assay can be performed. Both methods provide Kᵅ’s that are comparable to literature values. Existing detection methods use a radioactive filter binding assay that detects assay end points only, and have complicated disposal requirements. The proprietary masking dye ensures that fluorescence interference from compound libraries or media is minimized. This method of measuring transporter activity provides a new approach to screening for drug therapies addressing ailments such as depression, bipolar disorder, Parkinson’s, and Alzheimer’s disease.

**Introduction**
Norepinephrine, serotonin and dopamine are released from vesicles into the synaptic cleft, where they can diffuse and bind to pre- and postsynaptic receptors. Neurotransmitter transporters allow reuptake of these neurotransmitters for recycling by active co-transport down a Na⁺/Cl⁻ gradient. Thus neurotransmitter transporters regulate neuronal signaling by modulating neurotransmitter concentration in the synaptic cleft and are important targets for neuroscience drug discovery.

The assay described exploits the similarity of the norepinephrine, serotonin and dopamine reuptake transporters (NET, SERT and DAT) by using a fluorescent substrate mimetic for all three transporters.

**Assay Principle**
A proprietary fluorescent dye (Ex:440 nm; Em:520 nm) serves as substrate mimetic and is taken up into the cell specifically through DAT, SERT and NET. This dye, in combination with a proprietary masking dye, results in a homogeneous no-wash assay that can be read in real-time kinetic or endpoint mode, enabling mechanistic studies as well as HTS applications. Suitable instruments are fluorescence plate readers like Spectramax M5, Analyst GT, Flexstation, and FLIPR Tetra. Figure 2 shows the simple assay protocol.

**Kᵅ determination of fluorescent dye on SERT, DAT and NET**

\[ Kᵅ = \frac{V₅₀}{[S]} \]

Kᵅ for the fluorescent substrate mimetic used were determined for DAT, SERT and NET to be 1.85µM, 0.96µM and 0.33µM respectively (Figure 5).

**z’ factor determination for NET**

We evaluated the relationship of cell number to fluorescent dye coupled with a proprietary combination with a proprietary masking dye, results in a z’factor > 0.6 at all cell number conditions tested. The corresponding Ki’s were calculated using literature as shown in figure 9.

**Kinetic read example raw data: HEK-hSERT cells**

Figure 3: HEK cells stably expressing human SERT were plated ON at 10,000 cells per well in poly D-lysine-coated 384 well plates. Medium was removed and nisoxetine in HBSS-0.1% BSA was incubated with cells for 10 minutes at 37°C prior to dye addition. The assay was read on Molecular Devices’ Flexstation I instrument in kinetic mode for 30 minutes.

**PLATING OPTIONS**
Cells for the fluorescent neurotransmitter assay can be plated the day before or on the same day of the experiment. This increases the flexibility of assay planning, especially in an HTS setting. Figure 4 shows an inhibition curve with Neflinine on HEK NET cells.

**Figure 4: Comparison of same-day and overnight plating for HEK NET cells illustrated with a Neflinine inhibition curve. Response is expressed as area under the curve.**

**Figure 5: Kᵅ determination of fluorescent substrate mimetic for SERT, DAT, and NET.**

**Figure 6: Correlation between inhibition values obtained with the Neurotransmitter Transporter Uptake Assay and those found in literature for nine known inhibitors.**

**NET LOPAC screen: cherry pick**
Six inhibitors were selected from the library screen and their IC₅₀ for NET was determined. The resulting inhibition curves are shown in figure 8. The corresponding Kᵅ’s were calculated using a Kᵅ of 0.63µM determined for this particular NET cell line (data not shown) and a fluorescent substrate concentration of 2 µM.

**Table 2: Literature sources for inhibitors tested.**

**Figure 7: Scatter plot for LOPAC screen at 3µM compound on HEK-NET cells.**

**Correlation between inhibition values obtained with the Neurotransmitter Transporter Uptake Assay and those found in literature for nine known inhibitors.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Assay inhibition (%)</th>
<th>Literature inhibition (%)</th>
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<tbody>
<tr>
<td>Neflinine</td>
<td>85.2</td>
<td>82</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>63.1</td>
<td>60</td>
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<tr>
<td>Bupropion</td>
<td>92.5</td>
<td>90</td>
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<tr>
<td>Clomipramine</td>
<td>78.6</td>
<td>75</td>
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<tr>
<td>Imipramine</td>
<td>80.3</td>
<td>83</td>
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<tr>
<td>Sertraline</td>
<td>90.5</td>
<td>91</td>
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<tr>
<td>Protriptyline</td>
<td>70.2</td>
<td>68</td>
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<tr>
<td>Amoxapine</td>
<td>85.9</td>
<td>87</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>79.8</td>
<td>77</td>
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</table>

**Conclusions**
A novel fluorescence-based transporter assay has been developed that enables real-time analysis of DAT, NET or SERT uptake in a homogeneous, nonradioactive format.

- Either kinetic or endpoint mode can be used; the assay is scalable to 96 or 384 well plates.
- Kᵅ’s obtained is in good agreement with published Kᵅ values for DAT, NET and SERT antagonists.
- Due to the brightness of the fluorescent dye and its lack of non-specific uptake, z’ factors for this assay are robust using any of the Flexstation family of instruments or the M5 multimode reader from Molecular Devices.