Molecular Devices

Product Insert

Neurotransmitter Transporter Uptake Assay Kit

Product # R8173 (Explorer Kit)
R8174 (Bulk Kit)

About the Neurotransmitter Transporter Uptake Assay Kit

The homogeneous Neurotransmitter Transporter Uptake Assay Kit from Molecular Devices Corporation provides a fast, simple and reliable fluorescence-based assay for the detection of dopamine, norepinephrine and serotonin transporter (DAT, NET and SERT, respectively) activity in expressing cells. The kit utilizes a fluorescent substrate that mimics the biogenic amine neurotransmitters and is taken into the cell through those specific transporters, resulting in increased fluorescence intensity. In this convenient no-wash procedure, cells are incubated with the kit reagent and transferred to the plate reader for evaluation at the same time. The assay can be performed on any bottom-read mode fluorescence microplate reader in endpoint as well as real time kinetic mode.

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INTRODUCTION

Assay Principle

About the Neurotransmitter Transporter Uptake Assay Kit

At the center of the kit is a fluorophore that is mimicking biogenic neurotransmitters and is actively transported into the cell through the norepinephrine, dopamine and serotonine transporters. After incubation with a compound (e.g. inhibitor) the dye solution is added to the well and the fluorescent dye is transported into the cell. External fluorescence is extinguished with the masking dye. The fluorophor mimicking the neurotransmitters fluoresces when it enters the cell. Thus this assay can be run in a no-wash, kinetic mode. This allows the user to follow the transport of the dye through the expressed transporters in a real time mode. The Molecular Devices' homogeneous Neurotransmitter Transporter Uptake Assay is a flexible and simple mix-and-read protocol that can be performed in 96- or 384-well microtiter plates, rendering this assay ideal for high throughput screening applications as well as for follow-up mechanistic studies.

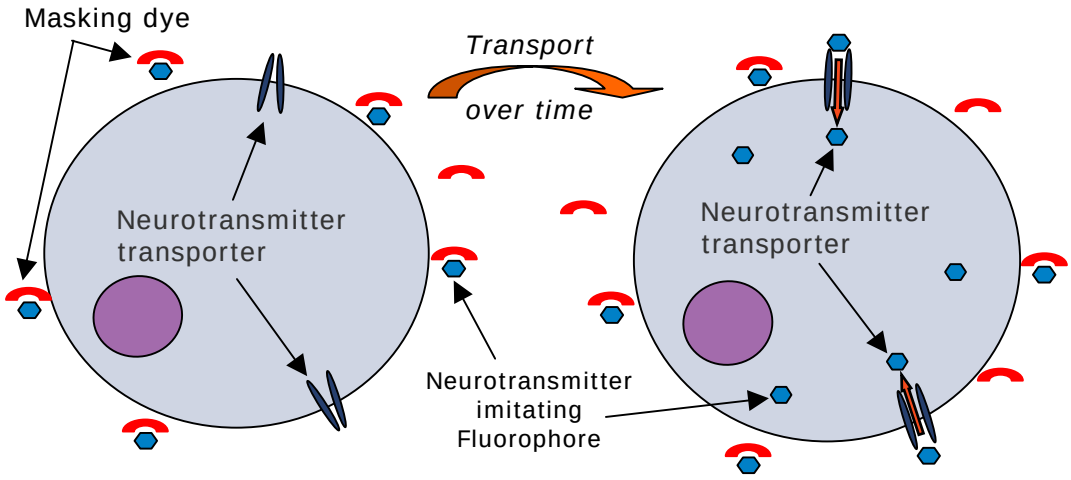


Figure 1: Neurotransmitter Transporter Uptake Assay principle

Advantages

Molecular Devices has developed the Neurotransmitter Transporter Uptake Assay, which not only eliminates the need to use radioactivity, but also offers additional advantages over existing assays:

- Flexible and simple mix-and-read procedure with little hands-on time
- High sample throughput enabling true HTS screening
- Minimal perturbation of the cells maximizing cell competence
- Can be read on any bottom-read fluorescence microplate reader
- Can be used in either 96-well or 384-well format
- Robust assay window, with high precision and Z' factors
- Endpoint and real-time kinetic read mode, enabling HTS application as well as mechanistic studies in the same cell system.

Applications

The kit has been designed for cells exogenously expressing the biogenic amine neurotransmitter transporters DAT, NET or SERT, providing a homogeneous assay for their transporting activity. The assay conditions were optimized for HTS applications. However, current studies also show promising results in regard to monitoring uptake activity of endogenous transporters.

MATERIALS & EQUIPMENT

Table 1: The Neurotransmitter Transporter Uptake Assay Kit contents

Kit Components

Kit	Description
Explorer Kit (R8173)	• 5 vials of lyophilized fluorescent dye/masking dye mix, each sufficient for one (1) 96-well or 384-well plate (5 plates total).
Bulk Kit (R8174)	• 5 vials of lyophilized fluorescent dye/masking dye mix, each sufficient for ten (10) 96-well or 384-well plates (50 plates total).

Table 2: Further Reagents and supplies needed but not part of the kit

Materials Required but Not Provided

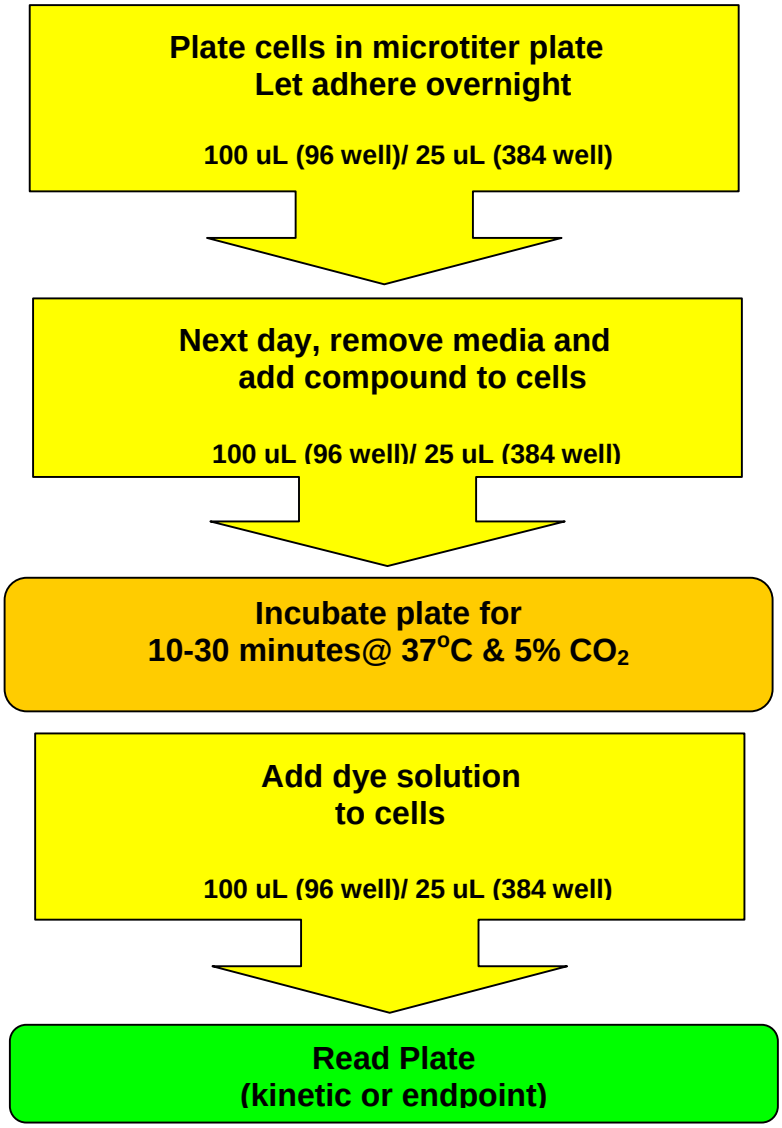
Item	Suggested Vendor
HBSS Buffer (1X Hank's Balanced Salt solution containing 20 mM HEPES	• 10X Hank's Balanced Salt Solution (#14065-056, Gibco or equivalent) • 1M HEPES buffer solution (#9319, Irvine Scientific or equivalent) • Water for cell culture (# 9312, Irvine Scientific or equivalent)
Bovine Serum Albumin (BSA)	• BSA, Fraction V, 35% Solution (A7979, Sigma)
Assay plates: 96- or 384- well black clear-bottom plates coated with poly-D-lysine (0.1 mg/mL) to enable cell adherence	• BD Biocoat plates (P/Ns 356640 or 356663), or similar

Storage and Handling

Upon receipt of the kit, store the Neurotransmitter Transporter Uptake Assay Kit at –20°C. Under these conditions the reagents are stable for six months in the original packaging.

After formulation, the Dye Solution is stable for up to eight hours at room temperature. Aliquots can be frozen at –20°C and stored for up to 5 days without loss of activity.

QUICK START PROTOCOL



DETAILED EXPERIMENTAL PROTOCOL

Cell Handling

The homogeneous Neurotransmitter Transporter Uptake Assay is designed to work in cells expressing neurotransmitter transporters such as HEK cells stably transfected with human dopamine transporter (hDAT), norepinephrine transporter (hNET) or serotonin transporter (hSERT). In this section, guidelines for how to plate and handle the cells are provided to perform the assay. The end user should optimize handling and plating conditions for their own particular cell line to be studied.

HEK-hDAT, HEK-hNET or HEK-hSERT cells can be prepared as described by Galli et al. (J Exp Biol. 1995 Oct;198(Pt 10):2197-212). These cells are maintained at 37°C, 5% CO_2 in monolayer culture in DMEM containing 10% FBS, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin, supplemented with 250 μg/mL G418. ***Note:*** **HEK-hSERT cells should be grown in dialyzed FBS, such as Gibco catalog #26400-036**.

Optimal cell plating conditions for the Neurotransmitter Transporter Uptake Assay requires a confluent cell monolayer before placing the plates in the fluorescent microplate reader.

Cells should be seeded at a density of 40,000-60,000 cells/well in a volume of 100µL for a 96-well plate and 12,500-20,000 cells/well in a volume of 25µL for a 384-well plate. It is recommended that cells are plated the night before and allowed to adhere/acclimate in the plate for 20 hours prior to assaying. Alternatively, cells can be plated on the same day as they are to be assayed as long as they have 3-4 hours to adhere/acclimate to the plate. Assay performance and precision is not noticeably compromised when the cells are plated on the same day.

Preparation of Dye Solution

The following procedure is designed for one 96- or 384-well plate using cells prepared as described above.

1. To prepare 1 L of 1X HBSS, pipet 100 mL of 10X Hank's Balanced Salt Solution and 20 mL of 1M HEPES buffer solution into 880 mL water for cell culture.

2. **a.** For the **Explorer Kit** (**R8173**), allow vial containing lyophilized fluorescent dye/masking dye mix to equilibrate to room temperature. Reconstitute by the addition of **10 mL** 1X HBSS. Mix by vortexing until contents of vial are completely dissolved. This is the Dye Solution.

   b. For the **Bulk Kit (R8174)**, allow vial containing lyophilized fluorescent dye/masking dye mix to equilibrate to room temperature. Reconstitute by the addition of 10 mL of 1X HBSS. Mix by vortexing until contents of vial are completely dissolved. Transfer the contents of the vial completely to a separate vessel by repeated additions of 1XHBSS and bring the total volume to **100 mL** with 1X HBSS. This is the **Dye Solution**.

Note: The components supplied are sufficient for proper dye incubation of the cells. For optimum results it is important to adhere to this protocol, without adding any additional reagents or altering volumes and concentrations.

Treatment of Cells with Test Compounds

1. To prepare 1X HBSS + 0.1% BSA Buffer, add 286 µL of 35% BSA per 100 mL of 1X HBSS. Optionally, the assay can also be run in the absence of BSA if there is a concern of the BSA interfering with the compounds effects

2. Remove plates from the incubator, aspirate the medium from the wells, and pipet 100 µL/well (for 96- well plates) or 25 µL/well (for 384-well plates) of compound diluted in 1X HBSS + 0.1% BSA Buffer to all wells. For blank wells, the compound diluent is added. Each well should now contain 100 µL volume.

3. Incubate at 37°C for 10 - 30 minutes, or a time period sufficient to allow binding of the compound to the transporter.

Dye Incubation

1. After incubation of the cells with compounds, add 100 µL (for 96-well plates) or 25 µL (for 384-well plates) of Dye Solution per test well.

2. Transfer the assay plate directly to a bottom-read fluorescence microplate reader for kinetic read-mode or incubate for 10-30 min at 37°C and then read for endpoint read-mode.

Note: For negative control wells, do not add Dye Solution but rather 1XHBSS. For further information, refer to **Data Analysis** section.

Running the Assay

1. For optimal measurement of this assay, we recommend Molecular Devices' Flexstation, Spectramax M5, Analyst GT or any of the other fluorescence intensity readers from MDC.
Recommended experimental setup parameters for the FlexStation, SpectraMax M5, and Analyst GT are shown in **Table 3.**

Table 3. Experimental setup parameters for FlexStation, SpectraMax M5, and Analyst GT

Parameters	FlexStation	SpectraMax M5	Analyst GT
Temperature setting	37°C	37°C	---
Excitation wavelength (nm)	440	440	425-35
Emission wavelength (nm)	520	520	510-20
Emission cut-off (nm)	515	515	505 dichroic (bottom)
PMT sensitivity	medium	medium	---
Reads/well	6	6	---
Attenuator	---	---	medium
Z-height	---	---	bottom of plate

2. The Neurotransmitter Transporter Uptake Assay can be read in either kinetic or endpoint mode:

- **For kinetic reading:** set up in bottom-read mode to read every 20-25 seconds for 96 well plates or 60-90 seconds for 384 well plates. We recommend at least 10-30 minutes total read time.

- **For endpoint reading:** set up in bottom-read mode to read at desired time point(s). We suggest that you wait at least 10 minutes after dye addition; a 30-60 minute time point may be more convenient. ***Note:*** store plates at 37°C in the dark during incubation after dye addition.

Data Analysis

1. For Z or Z' factor determination appropriate negative controls are imperative. Several approaches can be taken. For example wells containing cells without Dye Solution can be used as the negative control. Subtract the average of the negative control (no Dye Solution) wells from each well in order to obtain the transporter-specific fluorescent substrate uptake. The parental cell line, e.g., HEK, provides another example for a useful negative control for initial assay development. You may also want to add a potent and specific transporter inhibitor (such as nisoxetine for HEK-hNET cells, with a reported K_i = 5.1 nM) to triplicate wells containing cells, in order to provide a "maximally inhibited" signal. This can also serve as a negative control. A sample template showing a serial dilution of two drugs tested on HEK-hSERT and HEK parental cells is shown in **Figure 2** below.

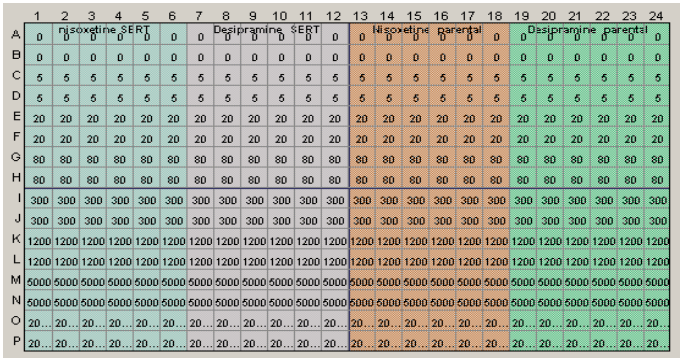


Figure 2. Sample assay template with drug dilutions (in nM) tested with HEK parentals and HEK-hSERT cells

2. If you are using SoftMaxPro® to analyze the data, we recommend checking the "Absolute Values" box in the Reduction window when reading in kinetic mode and graph the IC_{50}'s using "Area Under the Curve".

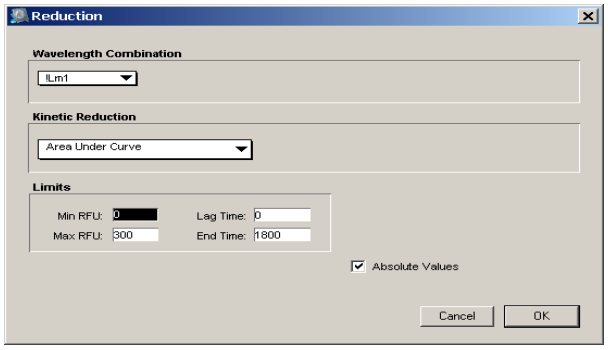


Figure 3. SoftMaxPro Reduction window for kinetic read

EXAMPLE DATA

SERT activity data: Raw data example

HEK cells stably expressing human SERT were plated O/N at 10,000 cells per well in poly D-lysine-coated 384 well plates. Medium was removed and nisoxetine (published K_i = 383 nM) in HBSS-0.1% BSA was incubated with cells for 10 minutes at 37°C prior to dye addition. The assay was performed according to the Experimental Protocol and read on Molecular Devices' Flexstation I instrument in kinetic mode for 30 minutes. Data is shown in **Figure 4.**

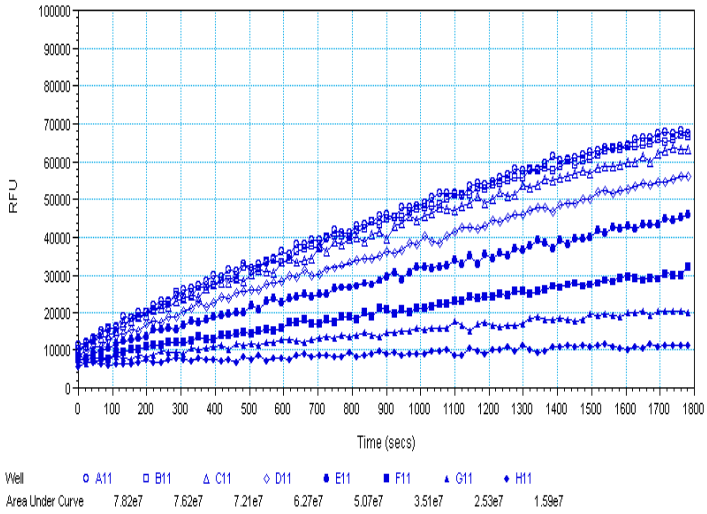


Figure 4. Nisoxetine inhibition in HEK-hSERT cells – raw FlexStation data with "Absolute Value" box checked.

NET activity Data: Plating Flexibility

HEK cells stably expressing human NET were plated O/N or on the day of assay at 20,000 cells per well in poly D-lysine-coated 384 well plates and allowed to adhere for 3hours. Medium was removed and nomifensine 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 III instrument in kinetic mode for 30 minutes. **Figure 5** shows the inhibition curve expressed as area under the curve

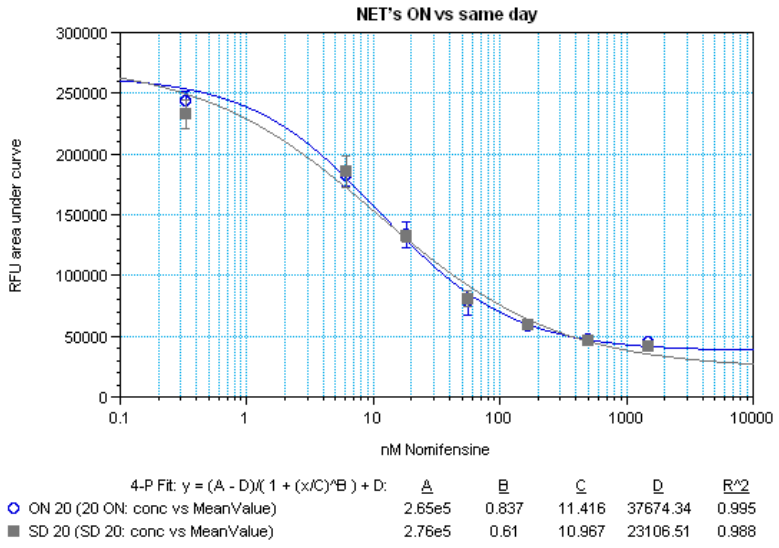


Figure 5. Comparison of over night (open blue circles) v.s. same day (filled black squares) plating of HEK-hNET cells (20.000 cells/well). Literature Ki values for Nomifensine on NET range from 6-30nM.

EXAMPLE DATA (continued)

IC_{50} correlation for SERT, NET, and DAT (Customer results)

Customer cells stably expressing human DAT, SERT or NET were plated O/N at 60.000 cells/well (96 well plate) and treated with 8 different known inhibitors. After 10 minutes the Dye Solution was added and the assay was run according the protocol described above. The log Ki was calculated from the resulting data using the Cheng-Prusoff equation and a correlation with known Ki values for all three transporters was determined. The correlation coefficients R^2 for each transporter is presented in the graph, the correlation coefficient (R^2 value) over all 24 values was 0.91.

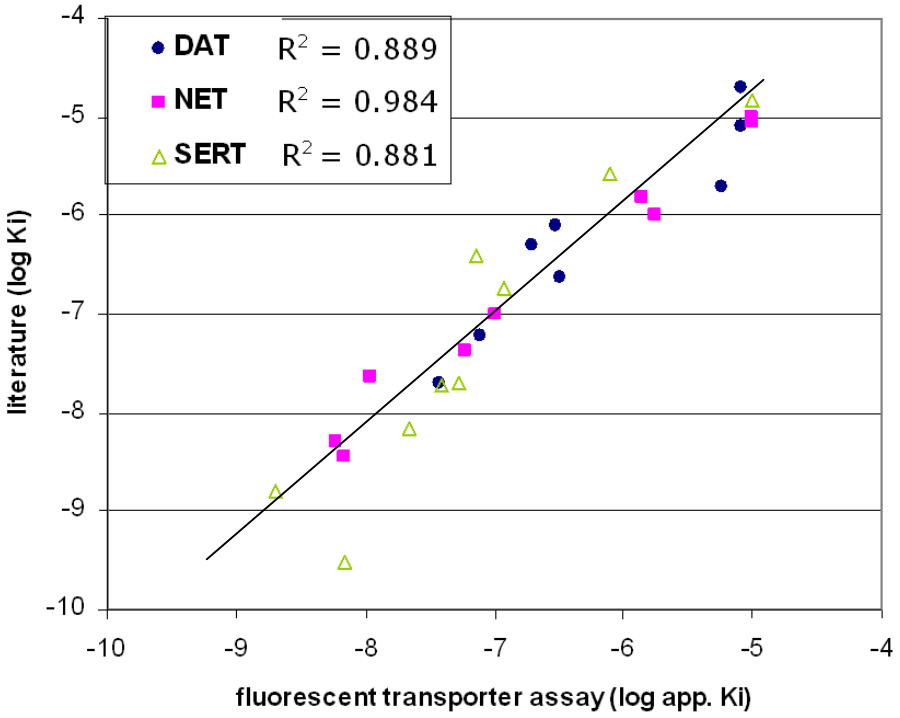


Figure 6. Scatter plot analysis correlating apparent Ki determined with the neurotransmitter transporter assay against identified literature values. The combined correlation coefficient R^2 value is greater than 0.9 indicating excellent correlation between literature and experimental values for this kit

USEFUL REFERENCES

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FREQUENTLY ASKED QUESTIONS

1. What influence does cell density have on the assay?
Cell density and plating can impact the performance as well as the signal readout. With the HEK based cell lines used in-house, we see an increase of signal intensity with increased cell number. However, beyond a certain point of plating density the standard deviations increase and thus the assay precision is reduced. The cell number recommended in this product insert was optimized for the cell lines available to MDC, other cell lines should be re-optimized in regard to these parameters. In addition we found that in overly confluent cells the IC_{50} of inhibitors can be right shifted.

2. Can I change the order of additions (dye solution before compound addition)?
As changing the order of addition (dye before inhibitor) will give reduced results, it is best to use the suggested guidelines for time incubations as listed in the procedures.

3. Do I need to add BSA to the HBSS for compound addition?
The assays perform similarly with and without BSA. We tend to include BSA in order to avoid binding of compounds to well walls or pipet tips.

4. What is the DMSO tolerance of the assay?
In our cell systems the assay is tolerant up to 3% DMSO (final concentration). However, DMSO results in a slight reduction of the maximal signal and thus should be present in vehicle control reactions.

5. Do I need to plate the cells the day before the assay?
No, if your cells settle down nicely within a reasonable time frame, the assay can be performed on the same day. In our hands, comparing similar cell numbers plated on the same day (allowed to adhere for 3 hours) versus o/n plating, showed no significant difference between these two conditions (see figure 5). Same day plating might be preferred in a HTS setting to enable 5 screening days/week.

Product Use Limitations and Warranty

Reagents may contain chemicals that are harmful. Due care should be exercised to prevent direct human contact with the reagent.

Use of this reagent includes a non-exclusive license to practice Bayer A.G., U.S. Patent 6,420,183 B1 and foreign counterparts thereof in conjunction with and only in conjunction with the use of this reagent from Molecular Devices Corp.

Molecular Devices Corporation makes no other warranties; either expressed or implied, including without limitation the implied warranties of merchantability and fitness for a particular purpose or use.

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