Molecular Devices

Threshold® Immunoassay System

ILA Application Note

# Contaminant bovine transferrin assay

## INTRODUCTION

Bovine transferrin, a 76,000 Dalton glycoprotein, is one of the constituents of bovine serum. Transferrin found in serum can be associated with iron ions. The iron-saturated form is called holo-transferrin, and the iron-poor form is called apo-transferrin. Regular serum contains all forms of transferrin at different degrees of saturation with iron. Fetal calf serum containing bovine transferrin can be used in mammalian cell culture media for the production of recombinant pharmaceuticals. During the purification process of a recombinant pharmaceutical, bovine transferrin may be co-purified with the product protein, and may represent a biological hazard for clinical use. Therefore, it may be necessary to assay contaminant bovine transferrin in a final product originating from mammalian cells. An assay for bovine transferrin can also be used to monitor the purification of the product throughout the process.

This application note provides a preliminary protocol and performance study of a bovine transferrin assay using a commercially available anti-bovine transferrin antibody. The note is intended as a guide and does not represent a validation of this assay, nor does it necessarily describe the optimal performance parameters.

## MATERIALS

1 ***Threshold® System*** from Molecular Devices Corporation (catalog #0200-0500), 1311 Orleans Drive, Sunnyvale, CA 94089, tel: 408-747-1700 or 800-635-5577.

2 ***Immuno-Ligand Assay Labeling Kit*** from Molecular Devices Corporation (catalog #R9002).

3 ***Immuno-Ligand Assay Detection Kit*** from Molecular Devices Corporation (catalog #R9003).

***Note: The Assay Buffer Concentrate included in the ILA kit is not used at any time in the contaminant human transferrin assay.***

4 ***BSA-free Assay Buffer.*** The Assay Buffer Concentrate provided in the ILA kit contains bovine serum albumin (BSA). Although the BSA used in the preparation of the buffer is of a high grade, it may contain contaminant transferrin that can affect the assay. An assay buffer without BSA is used in the human transferrin assay. The formulation for *BSA-free Assay Buffer* is:

| | 10X stock (one liter) | 1X final concentration |
|---|---|---|
| $KH_2P0_4$ | 4.08 g | 3.0 mM |
| $K_2HP0_4$ | 12.19 g | 7.0 mM |
| NaCl | 87.7 g | 150 mM |
| $NaN_3$ | 0.5 g | 0.005% (w/v) |
| Triton X-100 | 2.5 mL | 0.025% (v/v) |
| pH | 6.5 | 7.0 |

**Table 1:** Formulation for *BSA-free Assay Buffer.*

Prepare 500 mL of a 1:10 dilution of the 10X solution of *BSA-free Assay Buffer* with deionized water and filter through a 0.22 $\mu$m filter. Store 10X stock at 4°C. If crystals form, warm to room temperature to dissolve them before preparing the 1X solution. To prevent contamination of the buffer, use only individually wrapped sterile pipet tips and glassware rinsed copiously with deionized water.

5 ***Bovine transferrin*** (mixture of various forms) was purchased from Inter-Cell Technologies Inc., Hopewell, New Jersey, tel: 609-466-4766. Another source of bovine transferrin has been tested (apo and holo forms by JRH Biosciences): the different sources of transferrin are compared in "Characterization of the assay". The bovine transferrin from Inter-Cell Technologies will be used as the standard antigen in this application note, because it contains a mixture of forms of transferrin.

6 ***Sheep anti-bovine transferrin affinity purified polyclonal antibody*** from Bethyl Laboratories, Inc. (catalog # A10-122A) Montgomery, Texas, tel: 409-597-6111. Other sources of antibody have been tested (IgG fraction of rabbit anti-bovine transferrin from Inter-Cell Technologies, Inc. and IgG fraction of rabbit anti-bovine transferrin from Accurate Chemical & Scientific Corp.). The sheep antibody from Bethyl Lab provided the best response with the lowest background signal.

7 ***Ovalbumin*** from Sigma Chemical Co. (catalog #A-5503).

8 ***Sephadex® G-25 columns*** from Pharmacia Biotech (PD-10, catalog #17-0851-01).

9 ***Samples*** were kindly donated by customers of Molecular Devices Corporation.

## METHODS

### Labeling of antibody

The sheep anti-bovine transferrin antibody was labeled as described in the ILA section of the *Threshold System Operator's Manual* and the ILA application note *Optimizing the labeling of proteins*.

The sheep anti-bovine transferrin antibody is supplied in phosphate buffer containing preservatives. Therefore the antibody was dialyzed against PBS (10 mM Phosphate, 150 mM NaCl, pH 7.0) prior to labeling. DNP-biotin-NHS and fluorescein-NHS haptens were separately incubated with 250 µg of antibody, for two hours at room temperature, protected from the light. The molar coupling ratio (MCR) is defined as the number of moles of biotin or fluorescein incubated per mole of protein. The MCR used was 20:1 for the fluorescein and 30:1 for the biotin.

The unreacted hapten was separated from the antibody by passing the reaction mixture over a Pharmacia PD-10 column which had been equilibrated with 25 mL of PBS. The protein concentration, the protein recovery and the molar incorporation ratio (MIR) were calculated as described in the ILA section of the *Threshold System Operator's Manual*. The MIR is defined as the average number of moles of hapten covalently bound per mole of protein. Table 2 shows the molar incorporation ratios obtained for three independent labeling reactions. The stock concentration of the antibody was approximately 1 mg/mL.

| Labeling# | MIR Biotin/Ab | MIR Fluorescein/Ab |
|---|---|---|
| 1 | 3.8 | 3.6 |
| 2 | 3.1 | 3.5 |
| 3 | 3.0 | 3.6 |

**Table 2:** Molar incorporation ratios obtained for three labelings

After labeling, the antibodies were diluted to 20 µg/mL in *BSA-free Assay Buffer* with 1 mg/mL ovalbumin added as a carrier protein. Aliquots of 150 µL were stored at -20°C in Sarstedt sterile tubes.

### Determining the optimal concentration of antibodies per test (loading study)

Three concentrations of biotinylated and fluoresceinated anti-bovine transferrin antibodies were tested (20, 40 and 60 ng/test, with equal amounts of biotinylated and fluoresceinated antibodies per concentration tested) with three concentrations of bovine transferrin (0, 1 and 10 ng/mL) to evaluate the background rate and the "slope". The slope is a measurement of the change of signal as a function of change of antigen concentration. The sandwich format and a sequential incubation protocol were used (see the ILA section of the *Threshold System Operator's Manual*).

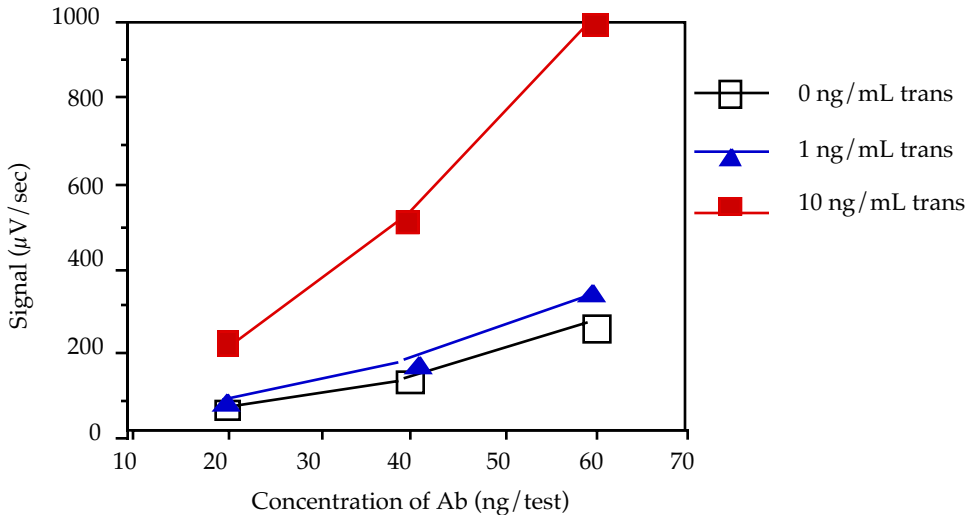


**Figure 1:** Loading study

Figure 1 shows that a concentration of 40 ng/test (400 ng/mL) of biotinylated antibody and 40 ng/test (400 ng/mL) of fluoresceinated antibody allowed acceptable background signal (with 0 pg/mL bovine transferrin) and satisfactory rates in the presence of bovine transferrin (1 and 10 ng/mL bovine transferrin). An antibody concentration of 40 ng/test was used for the remainder of the experiments in this application note.

### Assay protocol

**Step 1** Prepare the bovine transferrin standard curve in polypropylene tubes. Make serial dilutions of the bovine transferrin standard (2 to 3 mg/mL stock concentration for the bovine transferrin from Inter-Cell Technologies) in *BSA-free Assay Buffer*. The standards range from 100 ng/mL to 1 ng/mL of bovine transferrin. Suggested standard concentrations are 100, 50, 20, 10, 5, 2, 1 and 0 ng/mL of bovine transferrin. The transferrin standard concentration can be checked by a nonspecific protein assay such as BCA or a spectrophotometric measurement. The extinction coefficient at 280 nm of a 1% solution is approximately 11 for apo-transferrin and 14 for holo-transferrin. To determine the concentration of the transferrin from Inter-Cell, an average extinction coefficient of 12.5 was used.

**Step 2** If necessary, dilute the samples with *BSA-free Assay Buffer* in polypropylene tubes.

**Step 3** Prepare a mixture of the biotinylated and fluoresceinated antibodies, each at a concentration of 40 ng/test (400 ng/mL) in *BSA-free Assay Buffer* in a single polypropylene tube.

**Step 4** Dispense 100 µL of bovine transferrin standards and samples into polypropylene tubes.

**Step 5** Dispense 100 µL of the antibody combination prepared in Step 3 into the tubes using an Eppendorf Repeater Pipetter and a Combitip®.

**Step 6** Cover the tubes with Parafilm® and incubate for 2 hours at room temperature.

**Step 7** Reconstitute the Capture Reagent with 25 mL of *BSA-free Assay Buffer*. Prepare a 1:10 dilution of the reconstituted Capture Reagent with *BSA-free Assay Buffer* (1 volume of Capture Reagent + 9 volumes of *BSA-free Assay Buffer*). When the incubation is complete, dispense 1 mL of the diluted Capture Reagent into each tube with an Eppendorf Repeater Pipetter and a Combitip.

**Step 8** Transfer the reaction mixtures to the filtration units. The filter bases and filter blocks may be either new or re-used (see ILA Detection Kit package insert for cleaning instructions). Filter on low vacuum.

**Step 9** During the filtering step, reconstitute the Enzyme Reagent with 4 mL of *BSA-free Assay Buffer*. Prepare a 1:10 dilution of the reconstituted Enzyme Reagent (1 volume of Enzyme Reagent + 9 volumes of *BSA-free Assay Buffer*).

**Step 10** When the wells of the filtration units are empty, dispense 2 mL of Wash Buffer in each well, and filter on high vacuum. Turn off the vacuum.

**Step 11** Dispense 1 mL of diluted Enzyme Reagent into each well with an Eppendorf Repeater Pipetter and a Combitip and filter on low vacuum.

**Step 12** When the wells of the filtration units are empty, dispense 2 mL of Wash Buffer in each well, and filter on high vacuum. Turn off the vacuum and read the sticks.

## ASSAY CHARACTERIZATION

### Standard curve

The lowest calibrator of the standard curve (1 ng/mL) was selected by calculating the concentration of bovine transferrin that would generate a 30 to 50 $\mu$V/sec increase in signal over the background signal. This calculation was made based on the results of the loading study (see *Sandwich Assay Optimization* in the ILA section of the *Threshold System Operator's Manual*). The other calibrators of the standard curve were distributed over a range of 2 logs (100 fold) above the lowest calibrator. A quadratic equation best fits the data (see Figure 2).

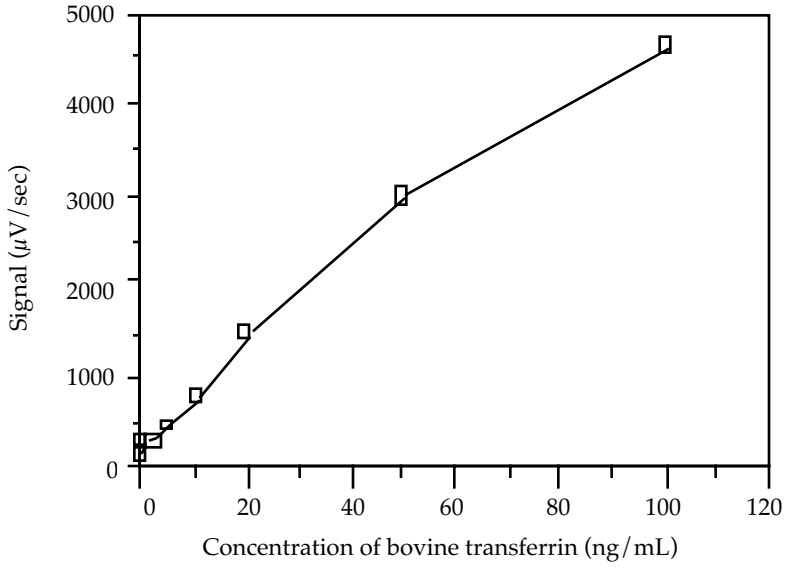


**Figure 2:** Bovine transferrin standard curve

Bovine transferrin from Inter-Cell was compared to the apo and holo-transferrin from JRH (see Figure 3). The stock concentrations were checked by spectrophotometric measurement. Transferrin from Inter-Cell and JRH were comparable at the low concentrations. At 50 ng/mL, JRH transferrin displayed signals 35% higher than Inter-Cell transferrin. The apo and holo forms of JRH transferrin gave a comparable response.

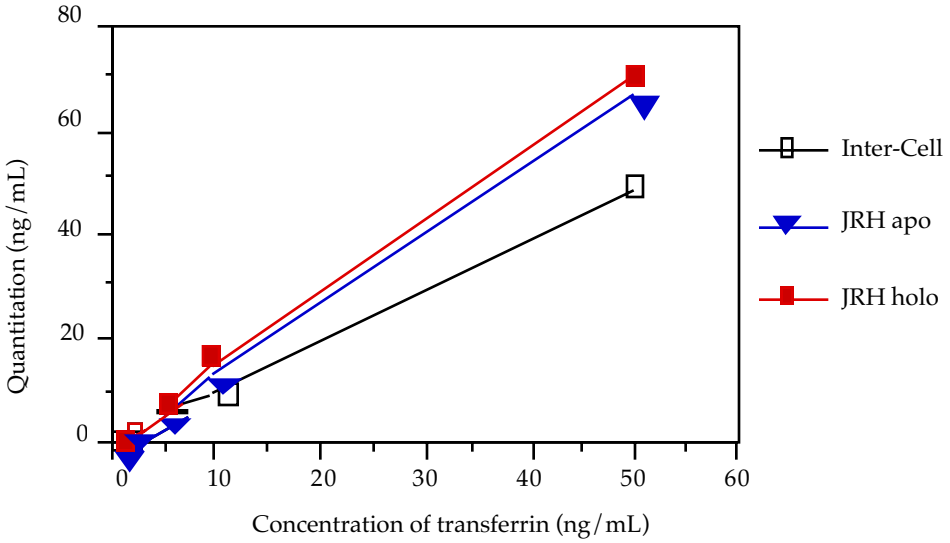


**Figure 3:** Comparison between three different transferrins

### Incubation time

A kinetic study was performed to determine the optimum incubation time. Four concentrations of bovine transferrin (20, 5, 1, and 0 ng/mL) were incubated with labeled antibodies for 4 hours, 2 hours or 1 hour.

The 4 hour, 2 hour and 1 hour incubations generated similar background signals. Figure 4 shows that an increase of slope was observed with an increase of the incubation time. The sensitivity of the assay can be improved by increasing the incubation time to more than 2 hours.

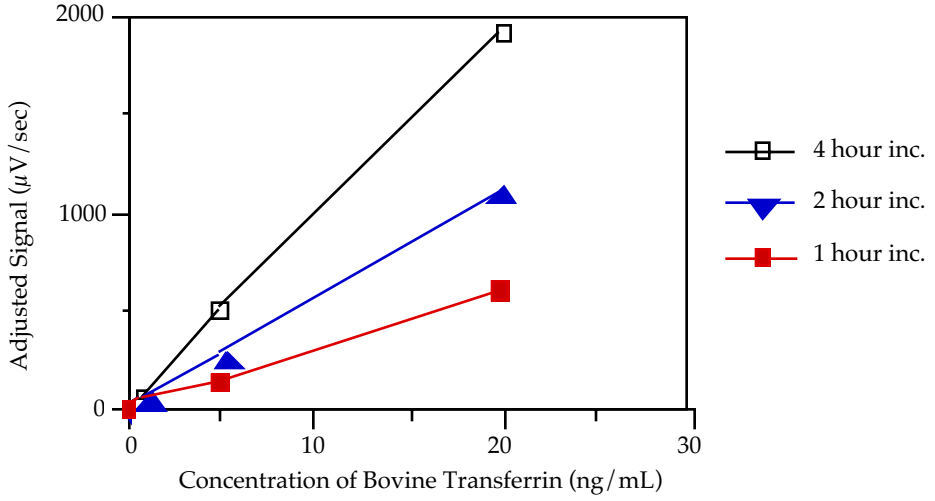


**Figure 4:** Effect of incubation time

### Limit of detection

See the *Threshold System Operator's Manual* for information on evaluating the limit of detection. This bovine transferrin assay typically allows detection of 1 ng/mL bovine transferrin with a 4 standard deviation separation from the background (data not shown).

## APPLICATIONS

The assay performance was evaluated by testing different protein samples for bovine transferrin content. The samples were tested non-spiked and spiked with a known concentration of bovine transferrin, and the percentage spike recovery (SR) was calculated as follows:

$$\frac{\textit{mean pg/mL spiked sample} - \textit{mean pg/mL non-spiked sample}}{\textit{mean pg/mL spiked buffer} - \textit{mean pg/mL non-spiked buffer}} \times 100 \; = \; \%SR$$

Acceptable spike recovery is defined as 100% ± 20%. Acceptable spike recovery means that the sample does not interfere in the measurement of the bovine transferrin and that the bovine transferrin quantitated in the sample is accurate.

### BSA samples

If BSA is used in the preparation of buffers or media, the transferrin concentration may be of interest. Three sources of BSA were tested for contaminant bovine transferrin. The original sample concentrations were 365 µg/mL (supplier #1), 713 µg/mL (supplier #2) and 937 µg/mL (supplier #3). All three BSA samples were tested non-spiked and spiked with approximately 15 ng/mL bovine transferrin. BSA solutions #1 and #2 were tested non-diluted and at a 1:2 dilution, BSA solution #3 was tested at $1x10^{-3}$ and $5 \times 10^{-3}$ dilutions.

| Sample | Spiked (ng/mL) | Non-spiked (ng/mL) | Net Spike (ng/mL) | Actual (ng/mg BSA) | % Spike Recovery |
|---|---|---|---|---|---|
| *BSA-free Assay Buffer* | 12.5 | 0.4 | 12.1 | | |
| BSA supplier #1 undiluted | 33.9 | 19.8 | 14.1 | 54.2 | 117% |
| 1:2 dilution | 23.8 | 9.6 | 14.2 | 52.6 | 117% |
| BSA supplier #2 undiluted | 19.7 | 8.2 | 11.5 | 11.5 | 95% |
| 1:2 dilution | 16.2 | 5.2 | 11.0 | 14.6 | 91% |
| BSA supplier #3 $1x10^{-3}$ dilution | 25.8 | 11.8 | 14.0 | 12,600 | 116% |
| $5 \times 10^{-3}$ dilution | 18.0 | 6.0 | 12.0 | 12,800 | 99% |

**Table 3:** Results obtained for the BSA solutions using the bovine transferrin assay protocol

The concentration of contaminant transferrin in the BSA solutions was 53.4 ng per mg of BSA for supplier #1, 13.1 ng per mg of BSA for supplier #2 and 12,700 ng per mg of BSA for supplier #3. The bovine transferrin assay allows the comparison of BSA purity from three different vendors.

**Fetal calf serum**

Fetal calf serum was tested for bovine transferrin. Quantitating bovine transferrin in fetal calf serum may be of interest if serum is used in the preparation of cell culture media. Each dilution tested ($2x10^{-5}$ to $1x10^{-7}$) was tested non-spiked or spiked with approximately 20 ng/mL of bovine transferrin.

| Sample | Spiked (ng/mL) | Non-spiked (ng/mL) | Net Spike (ng/mL) | Actual (ng/mL BSA) | % Spike Recovery |
|---|---|---|---|---|---|
| *BSA-free Assay Buffer* | 18.7 | 0.2 | 18.5 | | |
| $2x10^{-5}$ dilution | –* | 136.9 | – | – | – |
| $1x10^{-5}$ dilution | 71.3 | 47.6 | 23.7 | 4.8 | 128% |
| $5x10^{-6}$ dilution | 43.3 | 22.3 | 21.0 | 4.5 | 114% |
| $1x10^{-6}$ dilution | 21.8 | 5.3 | 16.5 | 5.3 | 89% |
| $2x10^{-7}$ dilution | 18.2 | 0.8** | 17.4 | – | 94% |
| $1x10^{-7}$ dilution | 20.1 | 0.9** | 19.2 | – | 104% |

**Table 4:** Results obtained for fetal calf serum solutions using the bovine transferrrin assay protocol. (*) signal too high to be quantitated off the standard curve. (**) below limit of detection.

The fetal calf serum tested allowed acceptable spike recovery at $5x10^{-6}$ and $1x10^{-6}$ dilutions, and based on data obtained from those dilutions, contained an average of 4.9 mg/mL of bovine transferrin.

**Murine IgG sample**

A murine $IgG_1$ sample was tested for contaminant bovine transferrin (undiluted and 1:2 dilution) with or without a spike of about 15 ng/mL bovine transferrin. The original sample concentration was 14 mg/mL murine $IgG_1$.

| Sample | Spiked (ng/mL) | Non-spiked (ng/mL) | Net Spike (ng/mL) | Calculated (pg/mg Mu $IgG_1$) | % Spike Recovery |
|---|---|---|---|---|---|
| *BSA-free Assay Buffer* | 12.7 | 0 | 12.7 | | |
| Murine $IgG_1$ undiluted | 24.5 | 10.1 | 14.4 | 721 | 113% |
| Murine $IgG_1$ 1:2 dilution | 18.3 | 4.7 | 13.6 | 671 | 107% |

**Table 5:** Results obtained for a murine $IgG_1$ sample using the bovine transferrrin assay protocol

The sample tested allowed satisfactory spike recovery and contained 696 pg contaminant bovine transferrin per mg of murine $IgG_1$, based on averaging the data from diluted and non-diluted samples.

**Assay precision**

The Murine IgG1 sample (14 mg/mL) was tested in triplicate on 5 different days to study the day to day reproducibility. The repeatability within the same experiment was studied by testing five replicates. The following table lists the results obtained.

| Within assay repeatability | | Day-to-day reproducibility | |
|---|---|---|---|
| Day | Quantitation (ng bov. transf/mL) | Day | Quantitation (ng bov. transf/mL) |
| 1 | 10.0 | 1 | 10.3 |
| | 10.7 | 2 | 10.1 |
| | 10.2 | 3 | 10.6 |
| | 9.4 | 4 | 9.6 |
| | 10.3 | 5 | 11.1 |
| Mean | 10.1 | Mean | 10.3 |
| Std. dev. | 0.5 | Std. dev. | 0.6 |
| C.V. | 4.7% | C.V. | 5.4% |

**Table 6:** Reproducibility and repeatability of the bovine transferrrin assay. Each value is the mean of three replicates.

## SUMMARY

Data in this application note were generated using a 2 hour incubation and 40 ng/test of each labeled anti-bovine transferrin antibody (except as noted). These conditions allow a dynamic range of 2 logs (100 fold), and a detection limit for bovine transferrin of 1 ng/mL. The assay may be adjusted to meet different requirements: the incubation time can be reduced for a faster assay, or increased for a more sensitive assay. Validation of assay performance should be determined for each product tested.

SALES OFFICE

*United States & Canada*
Molecular Devices
Tel. +1-800-635-5577
Fax +1-408-747-3601

*Brazil*
Molecular Devices Brazil
Tel. +55-11-3616-6607
Fax +55-11-3616-6607

*China*
Molecular Devices Shanghai
Tel. +86-21-6887-8820
Fax +86-21-6887-8890

*Germany*
Molecular Devices GmbH
Tel. +49-89/96-05-88-0
Fax +49-89/9-62-02-34-5

*Japan*
Molecular Devices Japan, Osaka
Tel. +81-6-6399-8211
Fax +81-6-6399-8212

Molecular Devices Japan, Tokyo
Tel. +81-3-5282-5261
Fax +81-3-5282-5262

*South Korea*
Molecular Devices Korea, LLC
Tel. +82-2-3471-9531
Fax +82-2-3471-9532

*United Kingdom*
Molecular Devices Ltd.
Tel. +44-118-944-8000
Fax +44-118-944-8001

www.moleculardevices.com