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ProductInformation

Automated Protocol for ANTI-FLAG[®] High Sensitivity, M2 coated 96-well plate Using the Sciclone ALH 3000 Workstation (Caliper Life Sciences)

ANTI-FLAG High Sensitivity, M2 coated 96-well plate P 2983

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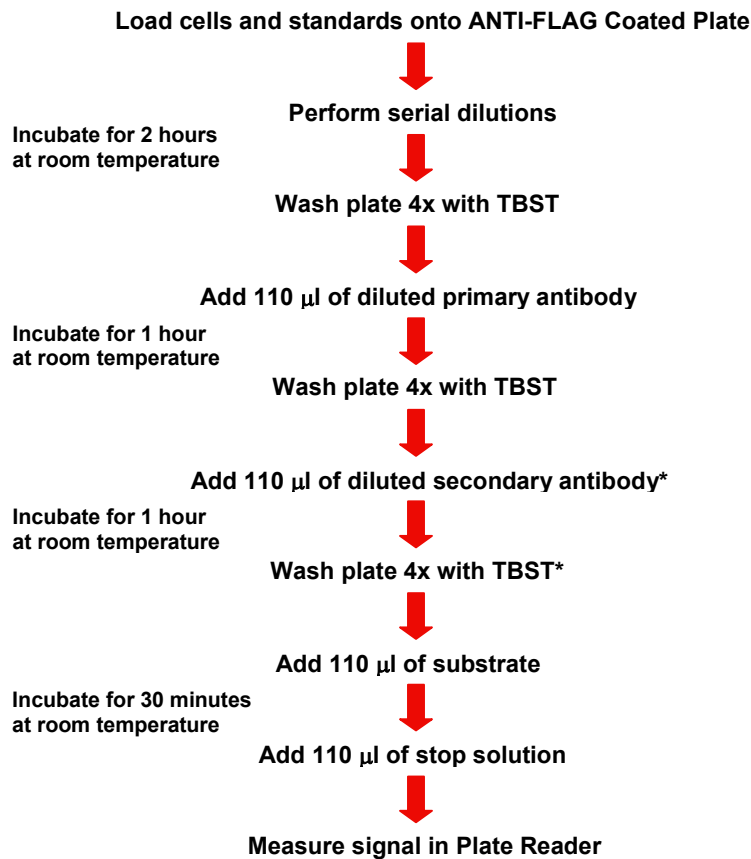
Automation Guide

I. Description

The ANTI-FLAG[®] High Sensitivity 96-well plates provide a convenient, high-throughput platform for the capture and detection of recombinant FLAG fusion proteins isolated from FLAG protein expression systems. The ANTI-FLAG M2 mouse monoclonal antibody is covalently linked to the surface of the multiwell plate via the Fc portion of the antibody. This linkage provides a favorable orientation of the antibody allowing for increased binding capacity. The ANTI-FLAG M2 coating can detect as little as 1 ng/well and has a capacity of up to 300 ng/well. In addition, the wells of the ANTI-FLAG High Sensitivity plates are pre-blocked with a complex solution containing bovine serum albumin. This combination provides significant timesavings for high-throughput users compared to assays where the antibody must be passively absorbed to the surface of a multiwell plate.

An automated method has been developed for ELISA applications using the ANTI-FLAG plate with the Sciclone ALH 3000 Workstation. The automated method is well suited for high-throughput applications including screening for expression and protein-protein interaction assays.

Automated ANTI-FLAG Plate ELISA Protocol



*Steps are optional if a conjugated primary antibody is used

II. Product Components

Product Code	P 2983 ANTI-FLAG® High Sensitivity, M2 coated 96-well plate
Package Size	1 ea 5 x 1 ea

III. Storage

The ANTI-FLAG High Sensitivity, M2 coated 96-well plates are stored at 2–8 °C. Prior to use, warm the plate to room temperature for 20 minutes.

IV. Materials to Be Supplied by the User

1. Cell lysates expressing a soluble FLAG fusion protein
2. Dilution Buffer compatible with the protein of interest and the antibodies used in the ELISA such as TBS-BSA (Sigma, T 6789)
3. Wash Buffer compatible with the antibody system of choice such as TBST (Sigma, T 9039)
4. Antibody** against the fusion protein of interest and a labeled secondary antibody, if necessary.
5. Substrate compatible with the label detection scheme of choice [3, 3', 5, 5'-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma, T 0440)]
6. If necessary the appropriate stop solution [TMB Stop Solution (Sigma, S 5814)]
7. Plate reader that is compatible with the substrate of choice.
8. 96-well reservoir with low profile and pyramidal bottom (Innovative Microplates, S30018)
9. 12-column reagent reservoir with high profile (Innovative Microplates, S30019)

**Since the ANTI-FLAG plate is coated with mouse monoclonal ANTI-FLAG Antibody, if a primary-secondary antibody system is used, the primary antibody should not be from mouse.

V. Cell Growth Requirements

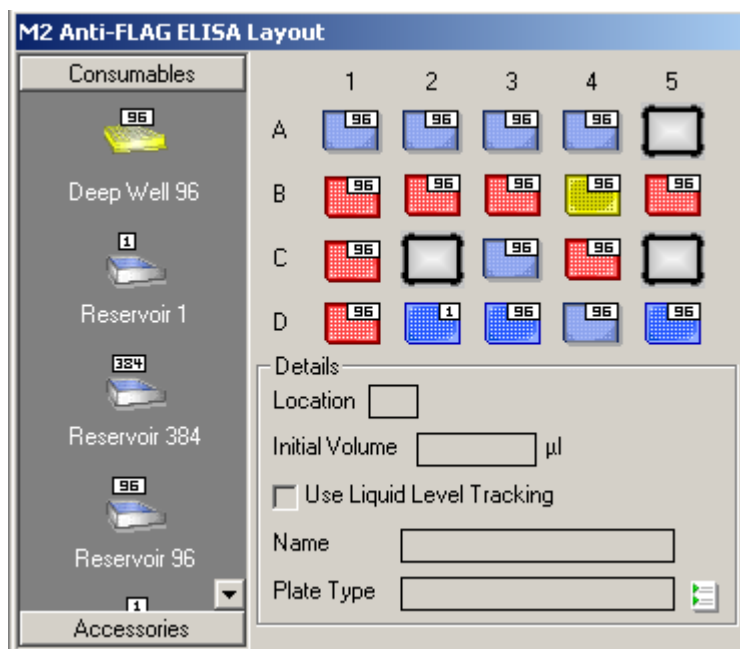
1. Grow cells expressing the recombinant FLAG fusion protein per standard procedures. ANTI-FLAG plates have been used successfully with cells grown in Terrific Broth.
2. Prepare cell lysates per standard procedures. Cell lysates have been successfully prepared using the CellLytic™ B Plus Kit (Sigma, CB 0500)

VI. Instrument Requirements for the Sciclone ALH 3000 Workstation

Part Description	Qty	Ordering Information
Deck Mounted Shaker	1	Contact Caliper
96-channel High Volume Head	1	Contact Caliper
Z8™ Pipettor	1	Contact Caliper
Gripper	1	Contact Caliper
I/O Box	1	Contact Caliper
Deck Locator	9	Contact Caliper
Tip Box Locator	7	#76523 (Caliper)
100 µl Disposable Tip Box	1	#68036 (Caliper)
200 µl Disposable Tip Box	6	#56362 (Caliper)
Bulk Dispense Module with Syringe Pump	1	Contact Caliper

VII. Deck Setup

Deck Layout for *M2 ANTI-FLAG ELISA* Method



Deck Position	Equipment
A1	96-well reservoir for substrate
A2	96-well reservoir for secondary antibody
A3	96-well reservoir for primary antibody
A4	96-well reservoir for stop solution
B1	200 µl tip box for handling the substrate
B2	200 µl tip box for handling the secondary antibody
B3	200 µl tip box for handling the primary antibody
B4	96-well deep well plate containing lysates
B5	200 µl tip box for stop solution
C1	200 µl tip box for handling dilution buffer
C3	96-well reservoir for diluent
C4	200 µl tip box for handling lysates
D1	100 µl tip box for dilutions
D2	Plate lid
D3	ANTI-FLAG High Sensitive, M2 plate
D4	12-column reservoir for waste
D5	Shaker

VIII. Reagent Preparation

1. *Cell Lysates*
 - a. If performing an ELISA using one lysate, add it to the 96-well reservoir located at position B4. Otherwise place a deep well plate containing multiple cell lysates at position B4.
2. *Standard*
 - a. Dilute the standard protein into the diluent of choice and place 100 μ l into wells A1, B1, and C1 of the ANTI-FLAG plate.
3. *Dilution Buffer*
 - a. The dilution buffer must be compatible with the protein of interest that is to be detected in an optimized ELISA. A TBS+BSA diluent, pH 8.0 (Sigma, T 6789) has been used on the ANTI-FLAG plates and is compatible with many proteins. To process a single plate of 96 samples, add 15 ml of the dilution buffer to the 96-well reservoir located at position C3.
4. *Wash Buffer*
 - a. Prepare a wash buffer that is compatible with the protein of interest and detection scheme of interest, and add this buffer to the designated bottle for the Bulk Dispense Module. A TBST wash buffer, pH 8.0 (Sigma, T 9039) has been used on the ANTI-FLAG plates and is compatible with many proteins.
5. *Primary and Secondary antibody solution*
 - a. Dilute the primary and secondary antibodies into at least 15 ml of diluent of choice, and add 15 ml of the mixture to the 96-well reservoir located at position A3 (primary antibody solution) and A2 (secondary antibody solution). Antibody dilutions should be prepared fresh.
6. *Stop solution*
 - a. Prepare the stop solution that is compatible with the detection scheme and substrate of interest. Add 15 ml of the mixture to the 96-well reservoir located at position A4. TMB stop solution (Sigma, S 5814) has been used on the ANTI-FLAG plate with TMB ELISA substrate.

IX. Automated Method Description

This overview describes the general liquid handling steps required to execute the automated procedure and can be customized for a variety of applications. For customized applications, see Section X.

A. General Procedures:

1. Set up deck layout: place the tip boxes, plates, and reservoirs at the appropriate positions on the deck as described in section VII.
2. Add reagents to the appropriate reservoirs as described in section VIII.
3. Turn the plate shaker module controller to the first black line after the "off" line (optional). If using the teleshake v1.2 software, use a setting of 550 rpm.
4. Run the method using Sciclone Software Version 3.2.
5. After running the automated method, read the plate on a plate reader compatible with the substrate of interest.

B. Sciclone Methods

1. *MAIN_ANTI-FLAG_ELISA*: This is the master program that performs all of the steps of the ELISA application including capture and detection of FLAG fusion proteins. All of the subsequent methods listed below are sub-routines that are called up and executed within this program
 - a. *SUB_Lysate*: Pipette mixes the cell lysates in the deep well plate and places the lysates into the ANTI-FLAG plate.
 - b. *SUB_Serial_Dilution_Standard*: Serially dilutes a standard from column 1 to column 11 (column 12 is blank).
 - c. *SUB_Serial_Dilution_Unknown*: Serially dilutes the unknown lysates from column 1 to column 11 (column 12 is blank).
 - d. *SUB_Standard_Unknown_Incubation*: Mixes the serially diluted samples on the shaker for 2 hours, and then aspirates them off.
 - e. *SUB_Primary_Antibody*: Dispenses the primary antibody of choice into all wells of the ANTI-FLAG plate and shakes the plate for 1 hour.
 - f. *SUB_Secondary_Antibody*: Dispenses the secondary antibody of choice into all wells of the ANTI-FLAG plate and shakes the plate for 1 hour.
 - g. *SUB_Substrate*: Dispenses the substrate of choice into all wells of the ANTI-FLAG plate and shakes the plate for 30 minutes.
 - h. *SUB_Stop_Solution*: Dispenses the stop solution into all wells of the ANTI-FLAG plate and shakes the plate for 10 seconds.
 - i. *SUB_Dilution_Solution_(TBS+BSA)*: Dispenses the dilution solution of choice into all dilution and "blank" wells of the ANTI-FLAG plate.
 - j. *SUB_TBST_Dispense*: Washes the plate 4 times with TBST.
 - k. *SUB_Deep_Aspiration*: After a TBST wash this is used to remove residual TBST.

MAIN_ANTI-FLAG_ELISA: Method Overview

Below is a brief summary of the *MAIN_ANTI-FLAG_ELISA* automated method. For complete program details download the automation program at www.sigmaaldrich.com/automation

1. Speed of Sciclone head movement is set at 80%.
2. Cell lysates (100 μ l) are pipette-mixed and transferred to wells D1, E1, F1, G1, and H1 of the ANTI-FLAG plate.
3. Serial dilutions are performed on standards and lysates.
 - a. 100 μ l of diluent is added to all wells of the ANTI-FLAG plate.
 - b. Standards are serially diluted 3-fold, and lysates are serially diluted 10-fold as described in plate map below. Between each dilution, samples are mixed 4 times.

Serial Dilution of Standards- Rows A, B, C

	1	2	3	4	5	6	7	8	9	10	11	12
A	Undiluted Standard	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	Blank
B	Undiluted Standard	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	Blank
C	Undiluted Standard	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	3-fold	Blank
D	Undiluted Lysate	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold
E	Undiluted Lysate	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold
F	Undiluted Lysate	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold
G	Undiluted Lysate	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold
H	Undiluted Lysate	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold	10-fold

Serial Dilution of Lysates- Rows D-H

4. The plate is incubated for 2 hours at room temperature while mixing on shaker.
5. The spent lysate is aspirated from the ANTI-FLAG plate and discarded.
6. 200 μ l of wash buffer is dispensed into each well of the ANTI-FLAG plate.
7. The ANTI-FLAG plate is shaken for 10 seconds and the wash buffer is aspirated from the plate.
8. Steps 6 and 7 are repeated a total of 4 times.
9. Primary Antibody (110 μ l) is dispensed into each well of the ANTI-FLAG plate.
10. The plate is incubated for 1 hour at room temperature while mixing on shaker.
11. The spent solution is aspirated from the ANTI-FLAG plate and discarded.
12. 200 μ l of wash buffer is dispensed into each well of the ANTI-FLAG plate.
13. The ANTI-FLAG plate is shaken for 10 seconds and the wash buffer is aspirated from the plate.
14. Steps 12 and 13 are repeated a total of 4 times.
15. Secondary Antibody (110 μ l) is dispensed into each well of the ANTI-FLAG plate.
16. The plate is incubated for 1 hour at room temperature while mixing.
17. The spent solution is aspirated from the ANTI-FLAG plate and discarded.
18. 200 μ l of wash buffer is dispensed into each well of the ANTI-FLAG plate.
19. The ANTI-FLAG plate is shaken for 10 seconds and the wash buffer is aspirated from the plate.
20. Steps 18 and 19 are repeated a total of 4 times.
21. Substrate (110 μ l) is dispensed into each well of the ANTI-FLAG plate.
22. The plate is incubated for 30 minutes at room temperature while mixing on shaker.
23. Stop buffer for the substrate (110 μ l) is dispensed into each well of the ANTI-FLAG plate.
24. The plate is incubated for 10 seconds at room temperature while mixing.

X. Method Customization

ELISA detection with primary-conjugate (no secondary)

It is possible to perform an ELISA with a labeled primary antibody conjugate (no secondary) by removing the steps in the method that call up the SUB_Secondary_antibody program and the subsequent SUB_TBST_Disperse.

XI. Performance Characteristics

Automated Method for the ANTI-FLAG ELISA Analysis: Standard Curve

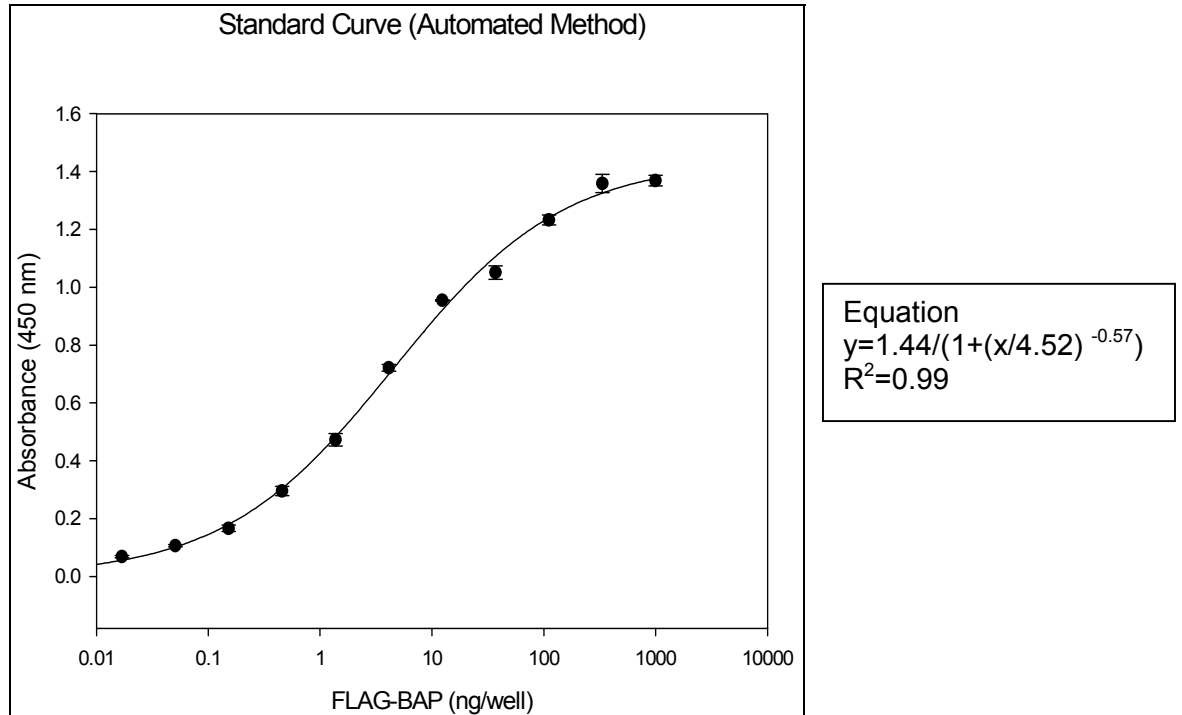


Figure 1. Standard curve generated from diluted FLAG-Bacterial Alkaline Phosphatase (BAP) standards. The automated Sciclone method was used to perform an ELISA. FLAG-BAP protein was serially diluted and then incubated at room temperature for 2 hours in the ANTI-FLAG M2 coated plate. For ELISA analysis, the BAP protein was first incubated with rabbit anti-alkaline phosphatase antibody for 1 hour at room temperature. Following four wash steps, the BAP protein was then incubated with the secondary antibody, a goat anti-rabbit IgG peroxidase conjugate, for 1 hour at room temperature. The peroxidase was detected by using a soluble TMB substrate for ELISA applications. The reaction was stopped using TMB stop solution. Absorbance was read on a Molecular Devices SpectraMax[®] Plus³⁸⁴ at 450 nm.

Automated Method for the ANTI-FLAG ELISA Analysis: Cross Contamination

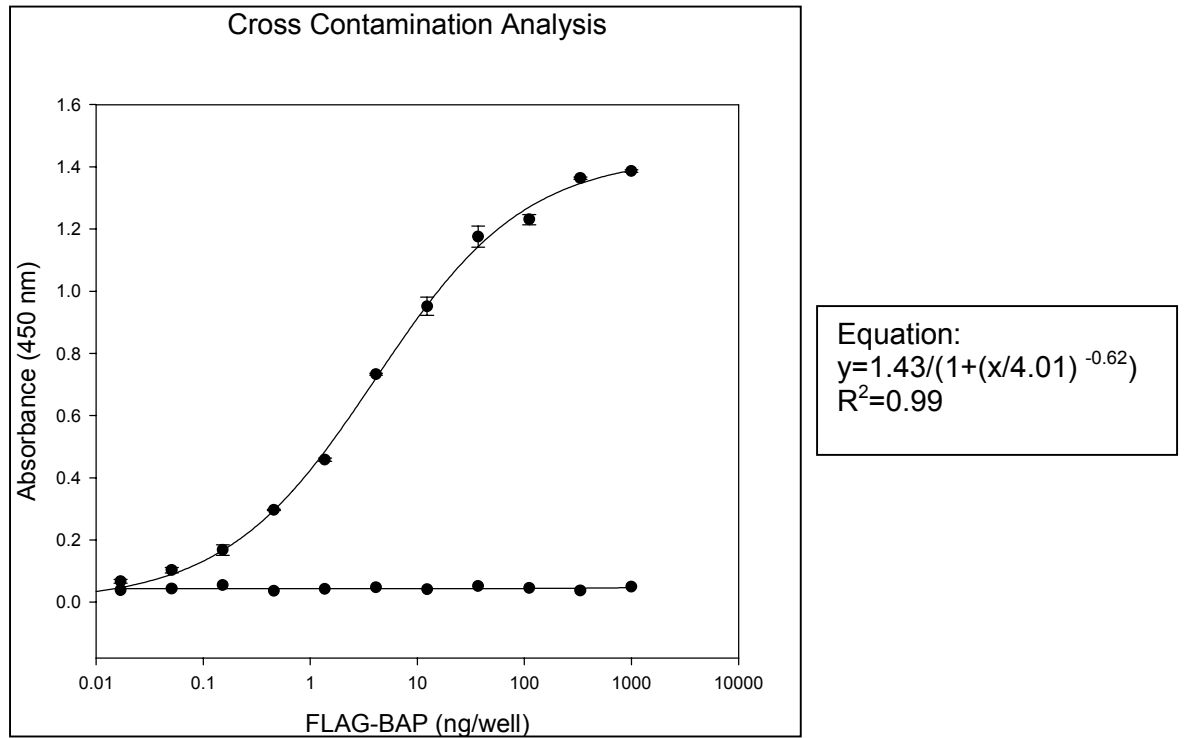


Figure 2. Cross contamination analysis using FLAG-BAP protein standard and blank wells. Cross contamination was evaluated in the automated ELISA method by inserting blanks between rows of FLAG-BAP protein standards. For ELISA analysis, the BAP protein was first incubated with rabbit anti-alkaline phosphatase antibody for 1 hour at room temperature. Following four wash steps, the BAP protein was then incubated with the secondary antibody, a goat anti-rabbit IgG peroxidase conjugate, for 1 hour at room temperature. The peroxidase was detected by using a soluble TMB substrate for ELISA applications. The reaction was stopped using TMB stop solution. Absorbance was read on a Molecular Devices SpectraMax[®] Plus³⁸⁴ at 450 nm. No cross contamination was detected in the blanks.

XII. Troubleshooting

Problem	Cause	Solution
The Standard Curve gave little or no signal, too much signal, or a non-linear signal.	The ELISA has not been optimized.	Optimize ELISA by changing the amount of primary and/or secondary antibody used in the assay.
	Substrate is not compatible with detection scheme.	The substrate must be compatible with the detection scheme of choice such as peroxidase or alkaline phosphatase. Check instrumentation for detection compatibilities. Check substrate stop solution, if used.
	A mouse primary antibody was used to detect the protein of interest.	If using secondary antibodies, do not use a mouse primary antibody to detect the protein of interest. The anti-mouse secondary antibody is detecting both the primary antibody and the Mouse M2 Anti-FLAG antibody bound to the plate.
	Others	Refer to the Technical Bulletin for the Anti-FLAG HS plate.
The unknown protein of interest was not detected.	The protein of interest was not expressed.	Confirm protein expression by Western blot using cell lysates.
	The protein of interest was expressed, but the FLAG sequence was missing or masked.	Confirm protein expression by Western blot using cell lysates and detection with anti-FLAG antibodies.

XIII. Contact Information

Technical Service
(800) 325-5832
email: techserv@sial.com

Customer Service
(800) 325-3010
(800) 588-9160
www.sigma-aldrich.com/order

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