

Technical Manual

RANSCREENER[®] EPIGEN Methyltransferase Assay

Product #: 3017-1K and 3017-10K

Transcreener® EPIGEN Methyltransferase Assay Kit

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1.0 Introduction

The Transcreener® EPIGEN Methyltransferase Assay is a universal biochemical HTS assay for enzymes that produce S-adenosylhomocysteine (SAH), including all enzymes within the histone (HMTs) and DNA (DNMTs) methyltransferase families. It combines the extensively validated Transcreener AMP²/GMP² Assay, which relies on fluorescent immunodetection of AMP, with coupling enzymes that convert SAH to AMP. Enzyme activity is signaled by a decrease in fluorescence polarization as the bound tracer is displaced from the Transcreener® AMP²/GMP² Antibody. The assay is a simple mix-and-read format with two liquid addition steps. Methyltransferase (MT) enzyme reactions are first quenched with Stop Buffer and then the SAH Detection Mixture containing coupling enzymes, Transcreener® AMP²/GMP² antibody, and tracer is added. The assay provides excellent signal at low substrate conversion, with a Z' ≥0.7 and ≥100 millipolarization shift (mP) under normal reaction conditions.

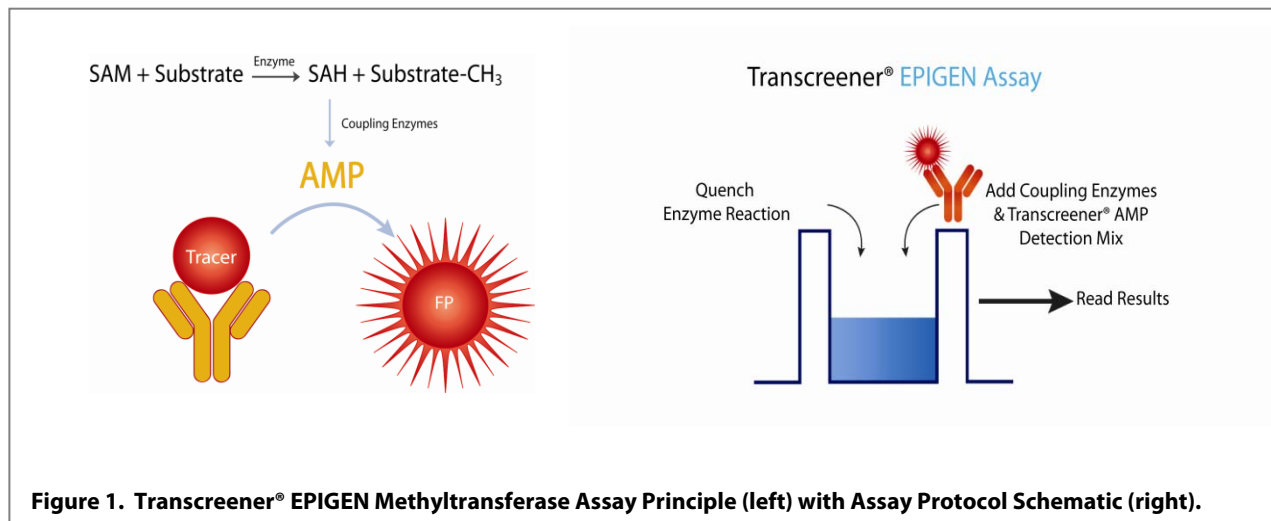


Figure 1. Transcreener® EPIGEN Methyltransferase Assay Principle (left) with Assay Protocol Schematic (right).

2.0 Transcreener® EPIGEN Methyltransferase Assay Components

Materials Provided

The kit contains sufficient reagents for 1,000 wells using concentrations up to 50 μM of S-Adenosylmethionine (SAM). Store reagents at -20°C or -80°C as indicated.

Component	Description
5 mM SAM	5 mM S-Adenosylmethionine (SAM). SAM supplied in the kit can be used for MT enzyme reactions and to create a SAM/SAH standard curve. If additional SAM is required, please contact BellBrook Labs for a list of preferred suppliers, as many commercial SAM products contain impurities that will decrease the assay window.
500 μM SAH	500 μM S-Adenosylhomocysteine (SAH). SAH is used to create a SAM/SAH standard curve.
Stop Buffer A, 1X	Stop Buffer A, 1X is an acidic reagent that is added directly to the reaction to inhibit MT activity.
Detection Buffer, 5X	Detection Buffer, 5X is used to prepare the SAH Detection Mixture. In addition to the Detection Buffer, the SAH Detection Mixture contains Coupling Enzymes 1 & 2, Cofactor, AMP ² /GMP ² Antibody, AMP ² /GMP ² AlexaFluor® 633 Tracer.
Coupling Enzyme 1, 1 mg/mL	Coupling Enzyme 1 is used to prepare the SAH Detection Mixture. This enzyme converts SAH to a substrate for Coupling Enzyme 2. Final concentration in the 20 μL volume is 2 $\mu\text{g/mL}$.
Coupling Enzyme 2, 1 mg/mL	Coupling Enzyme 2 is used to prepare the SAH Detection Mixture. This enzyme produces SAH from the Coupling Enzyme 1-generated substrate and Cofactor. Final concentration in the 20 μL volume is 1 $\mu\text{g/mL}$.
Cofactor, 5X	The Cofactor serves as a substrate for Coupling Enzyme 2.
AMP²/GMP² Antibody	A concentrated monoclonal AMP ² /GMP ² Antibody, provided in PBS with 10% glycerol. The concentration of AMP ² /GMP ² Antibody needed for a particular target enzyme depends on enzyme conditions particularly the initial [SAM].
AMP²/GMP² AlexaFluor® 633 Tracer, 800 nM	The AMP ² /GMP ² AlexaFluor® 633 Tracer is provided at 800 nM in 2 mM HEPES, pH 7.5 containing 0.01% Brij-35. The AMP ² /GMP ² AlexaFluor® 633 Tracer is at 4 nM at the time of polarization measurement (20 μL).

Materials Required

Ultrapure Water

Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, therefore reducing assay performance. Careful handling and use of ultrapure water eliminates this potential problem.

Enzyme Buffer Components

Necessary enzyme reaction components include enzyme, enzyme cofactors, substrates, and test compounds.

Plate Reader

A fluorescence polarization plate reader configured to measure fluorescence polarization of AlexaFluor® 633 is required. The Transcreener® EPIGEN Methyltransferase Assay using has been successfully performed on the following instruments: BMG Labtech PHERAstar, Perkin Elmer EnVision, and Tecan Safire². NOTE: Contact BellBrook Labs for additional instruments, information regarding instrument set up, and fluorescence polarization measurements.

Assay Plate

It is important that assay plates be entirely black with a non-binding surface. We recommend Corning® 384 well plates (catalog #3676).

Liquid Handling Devices

Use liquid handling devices that can accurately dispense a minimum of 2.5 μL into 384 well plates.



3.0 Protocol

The following protocol was developed for 384 well plate format with 15 μL enzyme reactions and 20 μL final volume at the time the plates are read. Use of the assay at different densities and well volumes will require proportional changes in reagent quantities.

3.1 Instrument Set Up

Determination of the optimal instrument settings for fluorescence polarization is essential to the success of the Transcreener[®] EPIGEN Methyltransferase Assay. Refer to Table 1 for filter sets and settings for common multimode plate readers. If you are using a different instrument, verify that it can measure fluorescence polarization (not simply fluorescence intensity). Contact BellBrook Labs if you have questions about settings and filter sets for a specific instrument.

PerkinElmer EnVision [®]		
Mirror	D658fp/D688 dual mirror	Part# 2100-4260
Excitation Filter/Bandwidth	620/40 nm	Part# 2100-5760
Emission Filter(P-pol)/Bandwidth	688/45 nm	Part# 2100-5790
Emission Filter(S-pol)/bandwidth	688/45 nm	Part# 2100-5780
BMG LABTECH PHERAstar Plus (Transcreener FP Optic Module)		
Excitation Filter/Bandwidth	590/50 nm	
Emission A Filter/Bandwidth	675/50 nm	
Emission B Filter/Bandwidth	675 nm	
Tecan Safire2 [™] (monochromator based)		
Excitation Wavelength/Bandwidth	635/20 nm	
Emission Wavelength/Bandwidth	670/20 nm	
Read-FP Mode		

EnVision is a registered trademark of PerkinElmer. Safire² is a trademark of Tecan.

Table 1. Instrument Filters & Settings. Filter settings and filter part numbers for commonly placed multimode plate readers. A complete list of instruments and instrument-specific application notes can be found online: www.bellbrooklabs.com/transcreener_instrument_validation.html.

i. Define the maximum polarization window for your instrument

Determine high (tracer + antibody) and low (free tracer) polarization values to define the maximum signal window for your specific instrument.

ii. Prepare High and Low polarization mixtures

High Polarization mixture (+ antibody): Add 2.5 μL of Stop Buffer A and 2.5 μL of SAH Detection Mixture (containing 80 $\mu\text{g}/\text{mL}$ AMP²/GMP² Antibody and 32 nM AMP²/GMP² AlexaFluor[®] 633 Tracer; see Table 3 for an example) to 15 μL enzyme reaction buffer devoid of enzyme and SAM.

Low Polarization mixture(- antibody): Add 2.5 μL of Stop Buffer and 2.5 μL of SAH Detection Mixture (containing 32 nM AMP²/GMP² AlexaFluor[®] 633 Tracer; see Table 3 for an example) to 15 μL enzyme reaction buffer devoid of enzyme and SAM.

iii. Measure the fluorescence polarization

The low and high polarization values should differ by >175 mP. Note that this measurement gives the maximal *signal window* that the instrument is capable of generating with these reagents. The *assay window* will be less than this, and will depend on how far the MT enzyme reaction proceeds. **If the signal window is <175 mP please contact BellBrook Labs for suggestions on optimizing the instrument filters or settings.**

3.2 Optimizing Enzyme and Detection Reaction Conditions

A polarization shift of 60-100 mP units and a Z' value of 0.5 indicates robust assay performance for HTS applications. For initial SAM concentrations of 5 μM , 10 μM and 50 μM , these criteria were achieved when less than 5% of the SAM was converted to SAH, whereas approximately 10% conversion was required at 1 μM and 0.5 μM initial SAM and 15% conversion and 30% conversion for 0.25 μM and 0.1 μM SAM respectively (Figure 3).

MT enzyme reaction conditions: The Transcreener® EPIGEN MT assay was designed for use with buffers and additives commonly used for MT enzymes. Run your enzymatic reaction with optimal buffer and additives at the requisite temperature. SAM concentration is critical, as it determines the AMP²/GMP² Antibody concentration (Table 2). The acceptor substrate (e.g., histone or histone-derived peptide) should be present at a concentration similar or higher than the SAM concentration to avoid non-linear kinetics resulting from substrate depletion.



Note: BSA at higher concentrations in MT reaction buffer may interfere with the assay resulting in a small assay window. Please contact us via phone (toll-free at 1-866-313-7881) or email (info@bellbrooklabs.com) for more information.

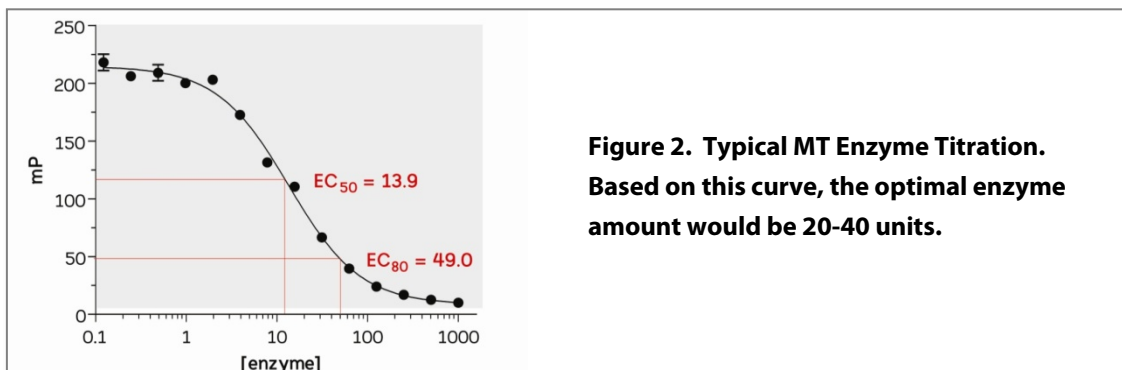
AMP²/GMP² Antibody concentration: This is usually the only assay component that requires adjustment for different reaction conditions. Its concentration will define the dynamic range of the assay, and it should be adjusted based on the SAM concentration used in the MT enzyme reactions. We have determined optimal AMP²/GMP² Antibody concentrations for five SAM concentration brackets up to 50 μM (Table 2). If you require SAM concentration outside of this range, please contact BellBrook Labs for recommendations.

SAM Concentration in 15 μL MT reaction	AMP ² /GMP ² Antibody Concentration in SAH Detection Mixture
**<1 μM	12 $\mu\text{g}/\text{mL}$
1 to 4 μM	20 $\mu\text{g}/\text{mL}$
5 μM to 9 μM	40 $\mu\text{g}/\text{mL}$
10 μM to 24 μM	60 $\mu\text{g}/\text{mL}$
25 μM to 50 μM	80 $\mu\text{g}/\text{mL}$

Table 2. Optimal concentration of AMP²/GMP² Antibody in SAH Detection Mixture for SAM concentrations up to 50 μM . **See Figure 3 for performance criteria at SAM concentrations <1 μM .

MT enzyme concentration: You will need to perform a pilot experiment to determine the optimal enzyme concentration to yield initial velocity conditions (substrate consumption $\leq 20\%$) and produce a sufficient assay window. The optimal enzyme concentration should be determined by serial

titration using the same conditions that will be used for screening or profiling. We recommend an enzyme concentration that produces 50% to 80% of the maximal change in polarization (EC_{50} to EC_{80}) with a polarization change of at least 100 mP (Figure 2). ($EC_{80} = (80/(100-80))^{(1/hillslope)} * EC_{50}$)



3.3 Reagent Preparation

Stop Buffer A: This is a low pH buffer that significantly inhibits the activity of many MTs. It is provided at the final concentration, ready to use (2.5 μ L per well, 20 μ L total reagent volume per well).

SAH Detection Mixture: Prepare on the day of use using amounts of each reagent as shown in Table 3 for 1,000 reactions (384 well density, 2.5 μ L SAH Detection Mixture per well, 20 μ L total reagent volume per well). Adjust quantities proportionately for fewer reactions and/or different plate densities and well volumes. The Stop Buffer A and SAH Detection Mixture are both stable for at least 16 hours at room temperature (20-25°C); we recommend keeping reagents cold as much of the time as possible.

SAM Concentration:	<1 μ M	1-4 μ M	5-9 μ M	10-24 μ M	25-50 μ M
Component	Volume				
Water	1315 μ L	1298 μ L	1257 μ L	1215 μ L	1173 μ L
Detection Buffer, 5X	500 μ L	500 μ L	500 μ L	500 μ L	500 μ L
Cofactor, 5X	500 μ L	500 μ L	500 μ L	500 μ L	500 μ L
AMP/GMP AlexaFluor® 633 Tracer, 800 nM	100 μ L	100 μ L	100 μ L	100 μ L	100 μ L
AMP ² /GMP ² Antibody, 1.2 mg/mL	25 μ L	42 μ L	83 μ L	125 μ L	167 μ L
Coupling Enzyme, 1 mg/mL	40 μ L	40 μ L	40 μ L	40 μ L	40 μ L
Coupling Enzyme 2, 1 mg/mL	20 μ L	20 μ L	20 μ L	20 μ L	20 μ L

Table 3. Recipe for SAH Detection Mixture for 1,000 wells of MT reactions using SAM concentrations up to 50 μ M. Final concentrations in the SAH Detection Mixture: 1X Detection Buffer, 1X Cofactor, 16 μ g/mL Coupling Enzyme 1, 8 μ g/mL Coupling Enzyme 2, 32 nM Tracer, and variable AMP²/GMP² Antibody.

3.4 Performing an Assay

The Transcreener® EPIGEN Methyltransferase Assay is designed for endpoint readout. It requires two liquid addition steps as the coupling enzymes are not stable in the acidic Stop Buffer A. Following incubation of MT enzyme reactions for the requisite time, Stop Buffer A and the SAH Detection Mixture are added in sequential 2.5 μ L aliquots, then reactions are mixed and allowed to equilibrate at room temperature for 90 minutes before reading.

Assay Steps:

- i. Run Enzyme Reaction (15 μ L).* Generally, a master mix containing all MT enzyme reaction components, except SAM, is dispensed into wells and reactions are started by adding SAM. Mix the plate, and incubate at the desired temperature and time.
- ii. Add Stop Buffer A (2.5 μ L).* The Stop Buffer A lowers the pH sufficiently to essentially stop most HMT reactions. It should be added to sequential wells using the same time interval used for initiation of enzyme reactions with SAM, and as soon as possible prior to addition of the SAH Detection Mixture.
- iii. Add SAH Detection Mixture (2.5 μ L).* Add as soon as possible following the Stop Buffer A, bringing the total volume to 20 μ L. Note that the MT enzyme reaction components (including SAM) will be diluted 1.3-fold and the components of Stop Buffer A and the SAH Detection Mixture will both be diluted 8-fold after this addition.
- iv. Read Plates.* Allow at least 90 minutes for the detection reaction to equilibrate before reading the plate. Our studies have indicated that the signal is stable (<10% change) for at least 24 hours at room temperature (20-25°C). If polarization measurement is to occur on the following day, seal the plates to prevent evaporation.

Control Reactions

When screening or profiling inhibitors, two types of controls are needed at a minimum: a) negative (“no enzyme”) control wells to calculate the decrease in polarization caused by enzyme activity; this control defines the upper limit (highest polarization value) of the assay window b) positive (“no inhibitor”) control wells lacking any test compounds to determine the full activity of the MT enzyme being screened. This control defines the lower limit of the assay window. Z' values are usually calculated from these two types of controls.

SAM/SAH Standard Curves

A standard curve (Figure 3) is needed to convert polarization values to product formation (SAH) for quantitative data analysis. (Note that because the Transcreener® EPIGEN MT Assay relies on a competitive binding reaction, the response is non-linear, so the signal is not directly proportional to reaction progress). Whether to use a standard curve is a matter of choice. Many Transcreener® users do not use one for screening purposes, because the criteria for a hit are based on statistical analysis of the raw data. However, if quantitative enzyme turnover information is required for Michaelis-Menten kinetic analysis, a standard curve will be needed.

The wells for the standard curve should contain all MT reaction components **except the MT enzyme** and receive the Stop Buffer A and SAH Detection Mixture. The curve is constructed to mimic an enzyme reaction: starting at the SAM concentration used for the screening reactions, SAM is decreased in increments and the SAH concentration is increased proportionately, keeping the sum of their concentrations [SAM + SAH] constant. We recommend using a twelve-point curve with concentrations of SAM and SAH corresponding to 0%, 1%, 3%, 5%, 7.5%, 10%, 15%, 20%, 30%, 40%, 60%, and 100% SAM conversion. Allow 90 minutes incubation prior to polarization measurement for the coupling enzyme reaction to reach completion.

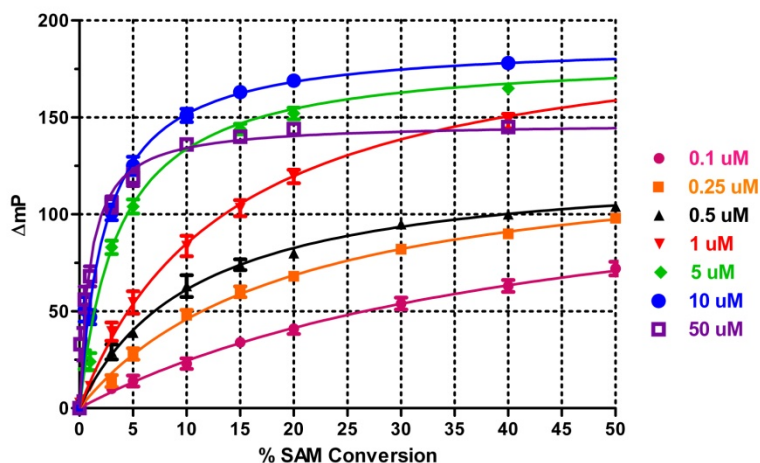


Figure 3. SAH/SAM Standard Curves. Sample data for standard curves starting at initial SAM concentrations of 0.1, 0.25, 0.5, 1, 5, 10, 50 μM are shown (SAM concentrations in the 15 μL mock enzyme reaction before the addition of Stop and Detection reagents). A polarization shift of 60-100 mP units and a Z' value of 0.5 indicates robust assay performance for HTS applications. For initial SAM concentrations of 5 μM , 10 μM and 50 μM , these criteria were achieved when less than 5% of the SAM was converted to SAH, whereas approximately 10% conversion was required at 1 μM and 0.5 μM initial SAM and 15% conversion and 30% conversion for 0.25 μM and 0.1 μM SAM respectively.

4.0 FAQs

How sensitive is the assay?

The lower limit of SAH detection is dependent on the starting concentration of SAM in the methyltransferase reaction. The assay becomes more sensitive with decreasing SAM concentration. For example, the lower limit of detection (LLD) for SAH in methyltransferase reactions utilizing 50 μM SAM is 0.25 μM SAH, whereas reactions containing 0.1 μM SAM has a SAH LLD of 0.01 μM . LLD is defined as the minimum amount of SAH that generates a $Z' > 0$.

What substrates can be used in the methyltransferase reaction?

Because the assay signal is independent of labeled or modified substrates and is based on the generic detection of SAH, peptides, native histones, and oligonucleotides, can all be used as acceptors of the methyl groups. Nucleosomes should also be acceptable substrates, but they have not been validated to date.

Does Stop Buffer A inhibit all methyltransferases? The activity of several histone and DNA methyltransferases have been inhibited by the addition of Stop Buffer A by lowering the pH of the reaction and by quenching EDTA with MgCl_2 . Note, if the target DNMT requires >25 mM EDTA, additional MgCl_2 may be necessary to stop the reaction.

Can this assay be run in continuous mode? This assay can be performed in real time by eliminating Stop Buffer A and including the SAH Detection Mixture components (coupling enzymes, cofactor, antibody, and tracer) in the methyltransferase reaction. However, this mode should only be used for relative activity comparisons because the extended signal equilibration time (1.5 hours) precludes accurate quantitation of SAH.

Does BSA interfere with the assay?

Bovine Serum Albumin (BSA) interferes with the detection reagents and should be avoided. Detergent such as Brij-35 can be substituted in the methyltransferase reaction to prevent non-specific binding of enzymes and substrates to the plate.

5.0 References.

Klink, T., Staeben, M., Twesten, K., Kopp, A., Kumar, M., Schall Dunn, R., Pinchard, C., Kleman, K., Klumpp, M., Lowery, R. Development and Validation of a Generic Fluorescent Methyltransferase Activity Assay Based on the Transcreener[®] AMP/GMP Assay. *Journal of Biomolecular Screening* **2011**, accepted for publication.

Terms & Conditions

U.S. Patent 7,332,278, 7,355,010 and 7,378,505 issued. U.S. Patent Application Nos. 11/353,500 and 11/958,965 and International Patent Application Nos. PCT/US04/002618 applied. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. BellBrook Labs LLC will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use, or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, BellBrook Labs LLC is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, BellBrook Labs LLC, 5500 Nobel Drive, Suite 250, Madison, Wisconsin 53711. Phone (608)443-2400. Fax (608)441-2967.

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