

TRANSCREENER[®] EPIGEN Methyltransferase Assay Technical Manual

Transcreener® EPIGEN Methyltransferase Assay Technical Manual

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U.S. Patent 7,332,278, 7,355,010 and 7,378,505 issued. U.S. Patent Application Nos. 11/353,500, 11/958,515 and 11/958,965, U.S. Divisional Application 12/029,932, and International Patent Application Nos. PCT/US07/088111, European Application Nos. 04706975.2 and 05785285.7, Canadian Application 2,514,877, and Japanese Application 2006-503179 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 other than use of the product or its components to provide a service, information, or data. Commercial Purposes means any activity by a party for consideration other than use of the product or its components to provide a service, information, or data 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 for therapeutic, diagnostic or prophylactic purposes; or (3) 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 230, Madison, Wisconsin 53711. Phone (608)443-2400. Fax (608)441-2967.

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1.0 Introduction

The Transcreener® EPIGEN Methyltransferase Assay is a far-red, competitive fluorescence polarization (FP) assay (**Figure 1**). The assay can be used with any enzymes that convert S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH). Examples include enzymes within the histone methyltransferase (HMT) and DNA methyltransferase (DNMT) families.

The Transcreener® assay is designed specifically for high-throughput screening (HTS), with a single-addition, mix-and-read format. It offers reagent stability and compatibility with commonly used multimode plate readers. The generic nature of the Transcreener® HTS assay platform eliminates delays involved in assay development for new HTS targets and greatly simplifies compound and inhibitor profiling across multiple target families.

The Transcreener® EPIGEN Methyltransferase Assay provides the following benefits:

- Accommodates SAM concentrations ranging from 0.1 μM to 50 μM .
- Excellent data quality ($Z' \geq 0.7$) and signal (≥ 100 mP polarization shift) at low substrate conversion using normal reaction conditions.
- Overcomes the need for time-consuming, one-off assay development for individual members within a group transfer enzyme family by using a single set of assay reagents that detect an invariant product.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

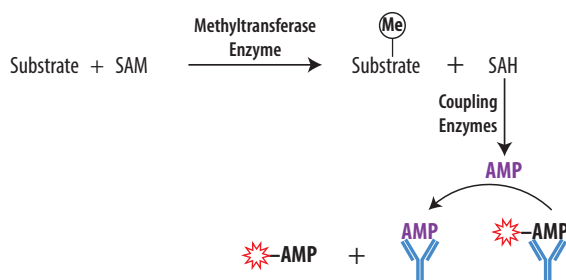


Figure 1. Schematic overview of the Transcreener® EPIGEN Methyltransferase Assay. The Transcreener® SAH Detection Mixture contains coupling enzymes that generate AMP from SAH, and an AMP/GMP Alexa Fluor® 633 tracer bound to an AMP²/GMP² antibody. AMP produced by the coupling reaction displaces the tracer, which rotates freely, causing a decrease in FP.

2.0 Product Specifications

| Product | Quantity | Part # |
|--|----------------|----------|
| Transcreener® EPIGEN Methyltransferase | 1,000 assays* | 3017-1K |
| FP Assay | 10,000 assays* | 3017-10K |

*The exact number of assays depends on enzyme reaction conditions. The kits are designed for use with 384-well plates, using 20 μL reaction volumes.

Storage

Store all reagents at -20°C or -80°C as indicated upon receipt.

2.1 Materials Provided

| Component | Composition | Notes |
|---|---|---|
| SAM | 5 mM | SAM supplied in the kit should be used for MT enzyme reactions and to create a SAM/SAH standard curve. |
| SAH | 500 μ M | 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. This reagent is used only to run the endpoint assays and can be eliminated for kinetic readouts. . |
| MT Detection Mix, 10X | 20 μ g/mL Coupling Enzyme 1, 10 μ g/mL Coupling Enzyme 2, 2.5 mM Cofactor, 40 nM AMP ² / GMP ² AlexaFluor® 633 Tracer, 100 mM HEPES, and 0.1% Brij-35. | The 10X Detection Mix should be diluted in nuclease-free water to 8X by adding the optimal antibody concentration to this mix for an endpoint assay. It can also be used to measure MT activity in a real-time assay by diluting it to 4X (without the stop reagent) in nuclease-free water. |
| AMP ² /GMP ² Antibody | 1.2 mg/mL solution in PBS with 10% glycerol* | The concentration of AMP ² /GMP ² Antibody needed for an enzyme target is dependent upon the SAM concentration and buffer conditions in the enzyme reaction (see Section 4.2). Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3017-1K) or 10,000 assays (Part # 3017-10K) at a SAM concentration up to 20 μ M. |

*The exact concentration may vary from batch to batch. Please refer to the Certificate of Analysis for an accurate concentration.



Note: Contact BellBrook Labs Technical Service for suppliers and catalog numbers for buffer components, and additional information regarding setup of FP instruments.

2.2 Materials Required but Not Provided

- **Ultrapure Water**—Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, reducing assay performance. Careful handling and use of ultrapure water eliminates this potential problem.
- **Enzyme**—Transcreener® EPIGEN Methyltransferase assays are designed for use with purified enzyme preparations. Contaminating enzymes, such as phosphatases or nucleotidases, can produce background signal and reduce the assay window.
- **Enzyme Buffer Components**—User-supplied enzyme buffer components include enzyme, enzyme cofactors, substrates, and test compounds.
- **Plate Reader**—A multidetection microplate reader configured to measure FP of the Alexa Fluor® 633 tracer is required. The Transcreener EPIGEN FP Assay has been successfully used on the following instruments: BMG Labtech PHERAstar; Perkin Elmer EnVision®; and Tecan Safire2™.
- **Assay Plates**—It is important to use assay plates that are entirely black with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4514). The suggested plate has a square well top that enables easier robotic pipetting and a round bottom that allows good Z' factors. It has a recommended working volume of 2–20 μ L.
- **Liquid Handling Devices**—Use liquid handling devices that can accurately dispense a minimum volume of 2.5 μ L into 384-well plates.

3.0 Before You Begin

1. Read the entire protocol and note any reagents or equipment needed (see Section 2.2).
2. Check the FP instrument and verify that it is compatible with the assay being performed (see Section 4.1).

4.0 Protocol

The Transcreener® EPIGEN Methyltransferase Assay protocol consists of 4 steps (Figure 2). The protocol was developed for a 384-well format, using a 15 μL enzyme reaction and 20 μL final volume at the time that the plates are read. The use of different densities or reaction volumes will require changes in reagent quantities.

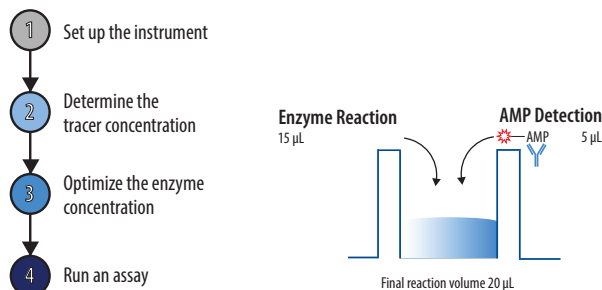


Figure 2. An outline of the procedure. The assay consists of 4 main steps with a mix-and-read format.

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for FP is essential to the success of the Transcreener® EPIGEN Methyltransferase Assay.

4.1.1 Verify That the Instrument Measures FP

Ensure that the instrument is capable of measuring FP (not simply fluorescence intensity) of Alexa Fluor® 633.

4.1.2 Define the Maximum FP Window for the Instrument

Measuring high (tracer + antibody) and low (free tracer) FP will define the maximum assay window of your specific instrument. Prepare High and Low FP Mixtures in quantities sufficient to perform at least 6 replicates for each condition.

Use MT Detection Mix and Stop Buffer A at 0.25X concentration in a 20 μL final reaction volume. This mimics the 4-fold dilution when adding 5 μL of detection mixture to 15 μL of an enzyme reaction.

High FP Mixture

Prepare the following solution:

| Component | Stock Concentration | Final Concentration | Example: 25 Assays | Your Numbers |
|---|---------------------|----------------------------|---------------------------------------|--------------|
| AMP ² /GMP ² Antibody | 1.2 mg/mL | 10 $\mu\text{g}/\text{mL}$ | 4.2 μL | |
| Stop Buffer A, 1X | 1X | 0.25X | 62.5 μL | |
| MT Detection Mix, 10X | 10X | 0.25X | 12.5 μL | |
| Water | | | 420.8 μL | |
| Total | | | 500.0 μL | |

The assay window will depend upon your initial SAM concentration. These volumes can be adjusted for fewer assays and different SAM concentrations.



Note: A complete list of instruments and instrument-specific application notes can be found online at: <https://www.bellbrooklabs.com/technical-resources/instrument-compatibility> Contact BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.



Low FP Mixture

Prepare the following solution:

Caution: Contact BellBrook Labs Technical Service for assistance if the assay window is <150 mP.

| Component | Stock Concentration | Final Concentration | Example: 25 Assays | Your Numbers |
|-----------------------|---------------------|---------------------|--------------------------------|--------------|
| Stop Buffer A, 1X | 1X | 0.25X | 62.5 μ L | |
| MT Detection Mix, 10X | 10X | 0.25X | 12.5 μ L | |
| Water | | | 425.0 μ L | |
| Total | | | 500.0 μL | |



Note: Contact BellBrook Labs Technical Service for assistance if you require nucleotide concentrations outside this range.

4.1.3 Measure the FP

Subtract the Low FP Mixture readings from the corresponding High FP Mixture readings. The difference between the low and high FP values should be >150 mP.

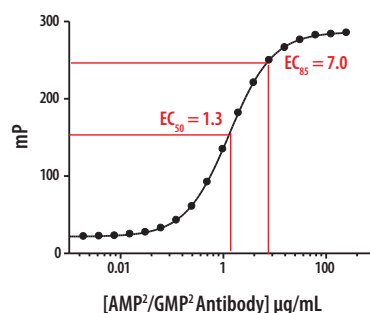
4.2 Determine the Optimal AMP²/GMP² Antibody Concentration

The antibody is 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 enzyme reactions. To produce the most sensitive and robust assay signal, it is necessary to perform an AMP²/GMP² Antibody titration in the buffer system ideal for your enzyme or drug target.

4.2.1 Titrate the AMP²/GMP² Antibody

1. Prepare the reaction buffer: 50 mM Tris-HCl (pH 8.5), 5 mM MgCl₂, 4 mM DTT, 0.01% Triton X-100, and 0.1 M NaCl. Include SAM and substrate but omit the enzyme.
2. Dispense 10 μ L of the reaction buffer into each well of columns 2–24.
3. Dispense 20 μ L of AMP²/GMP² Antibody (at 1 mg/mL in the same reaction buffer) into each well of column 1.
4. Remove 10 μ L from each well of column 1 and add it to the corresponding well of column 2.
5. Repeat step 4 for the remaining columns, thereby performing a 2-fold serial dilution across the plate to column 24.
6. Add 2.5 μ L of MT Detection Mix, 10X to each well.
7. Mix the plate, equilibrate at room temperature for 1 hour, and measure FP.

Figure 3. Antibody titration curve. The ideal range of antibody concentrations is shown in red.



4.3 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal MT enzyme concentration for the Transcreener® EPIGEN Methyltransferase Assay. Use enzyme buffer conditions, substrate, and nucleotide concentrations that are optimal for your target enzyme. 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 nonlinear kinetics resulting from substrate depletion. SAM concentration and the acceptor substrate concentration is critical, as it determines the AMP²/GMP² Antibody concentration (see Section 4.2). If a compound screen is planned, you should include the library solvent at its final assay concentration. Use enzyme buffer that provides optimal reaction conditions for your target enzyme. Run the enzymatic reaction at its requisite temperature and time period.

4.3.1 Enzyme Titration Steps

To achieve the most robust assay and a high signal, the quantity of enzyme required to produce a 50–80% change in FP signal is ideal (EC_{50} to EC_{80}) for screening of large compound libraries and generating inhibitor dose-response curves (see **Figure 3**). To determine the EC_{80} enzyme concentration, use the following equation:

$$EC_{80} = (80 \div (100 - 80))^{(1 \div \text{hillSlope})} \times EC_{50}$$

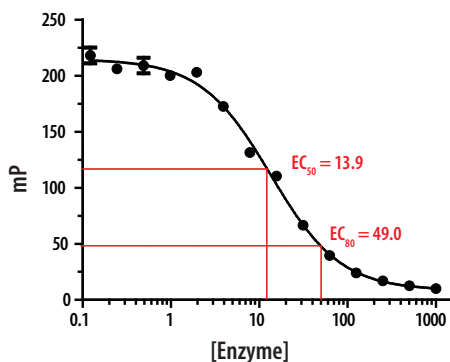


Figure 4. Enzyme titration curve. The ideal range of enzyme concentrations is shown in red.

4.3.2 Enzyme Assay Controls

The enzyme reaction controls define the limits of the enzyme assay.

| Component | Notes |
|-----------------------------|---|
| 0% SAM Conversion Control | This control consists of the MT Detection Mixture, the enzyme reaction components (without enzyme), and 100% SAM (0% SAH). It defines the upper limit of the assay window. |
| 100% SAM Conversion Control | This control consists of the MT Detection Mixture, the enzyme reaction components (without enzyme), and 100% SAH (0% SAM). It defines the lower limit of the assay window. |
| SAM/SAH Standard Curve | Although optional, a SAM/SAH standard curve can be useful to ensure day-to-day reproducibility and that the assay conditions were performed using initial rates. It can also be used to calculate product formed and inhibitor IC_{50} values. See Section 7.1 for a description of how to run the standard curve. |
| Background Control | Use only 0.5X enzyme reaction conditions and Stop Buffer A. |

4.4 Prepare Reagents

4.4.1 Stop Buffer A

Stop Buffer A is a low pH buffer that significantly inhibits the activity of many MTs. It is provided at the final concentration, ready to use. Each well will use 2.5 μ L SAH Detection Mixture for endpoint assays, with a total reagent volume of 20 μ L per well. Stop Buffer A is not required for kinetic assays.

4.4.2 SAH Detection Mixture

On the day you plan to run an assay, prepare SAH Detection Mixture using the amounts of each reagent shown below for 1,000 reactions (384 well plate). Each reaction will use 2.5 μ L of SAH Detection Mixture for endpoint assays or 5 μ L of SAH Detection Mixture for kinetic assays, with a total reagent volume of 20 μ L per well. Adjust quantities proportionately for fewer reactions and/or different plate densities and well volumes.



Note: 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 (on ice or at 4°C) until they are used. Stop Buffer A 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.

| Component | Endpoint Assay | Kinetic Assay |
|--|-------------------------|-------------------------|
| MT Detection Mix, 10X | 800 µL | 800 µL |
| AMP ² /GMP ² Antibody, 1.2 mg/mL | 8 × [EC ₈₅] | 4 × [EC ₈₅] |
| Water to Total Volume | 1,000 µL | 2,000 µL |

4.5 Run an Assay

4.5.1 Endpoint Assay

The Transcreener® EPIGEN Methyltransferase Assay is designed for endpoint readout. It requires 2 liquid addition steps, because the coupling enzymes are not stable in the acidic Stop Buffer A. Before you begin, prepare a master mix containing all enzyme reaction components except SAM.

1. Add the enzyme reaction master mix (without SAM) to test compounds and mix on a plate shaker.
2. Start the reaction by adding SAM, then mix. The final volume of the enzyme reaction mixture should be 15 µL. Incubate at a temperature and time ideal for the enzyme target.
3. Add 2.5 µL of Stop Buffer A:
4. Add 2.5 µL of SAH Detection Mixture (prepared in **Section 4.4.2**) to the 15 µL enzyme reaction. Mix using a plate shaker.
5. Incubate at room temperature (20–25°C) for at least 90 minutes and measure FP.



Caution: When running the assay in kinetic mode, make sure the enzyme reaction buffer contains MgCl₂. We recommend using 5 mM MgCl₂. Contact BellBrook Labs Technical Service for more information on buffer components.

4.5.2 Kinetic Assay

The Transcreener® EPIGEN Methyltransferase Assay can be run in kinetic mode to measure MT activity in real time. Before you begin, prepare a master mix containing all enzyme reaction components except SAM.

1. Add the enzyme reaction master mix (without SAM) to test compounds and mix on a plate shaker.
2. Start the reaction by adding SAM, then mix. The final volume of the enzyme reaction mixture should be 15 µL. Incubate at a temperature and time ideal for the enzyme target.
3. Add 5 µL of SAH Detection Mixture (prepared in **Section 4.4.2**) to the 15 µL enzyme reaction. Mix using a plate shaker.
4. Incubate at the required temperature and read the plates every 30 minutes for 3 hours, up to an overnight read. Seal the plates between measurements to prevent evaporation

4.5.3 SAH Detection Controls

These controls are used to calibrate the FP plate reader and are added to wells that do not contain enzyme.

| Component | Notes |
|--------------------------------------|--|
| Minus Antibody (Free Tracer) Control | This control contains the MT Detection Mix without the AMP ² /GMP ² Antibody and is set to 20 mP. |
| Minus Tracer Control | This control contains the AMP ² /GMP ² Antibody without the MT Detection Mix and is used as a sample blank for all wells. It contains the same AMP ² /GMP ² Antibody concentration in all wells. |

5.0 General Considerations

5.1 Reagent and Signal Stability

The Transcreener® technology provides a robust and stable assay method to detect MT activity.

5.2.1 Signal Stability

The stability of the FP assay window at 10% substrate conversion was determined after the addition of the SAH Detection Mixture to the standard samples. The FP assay window at 10% substrate conversion (10 µM) remained constant (<10% change) for at least 24 hours at room temperature (20–25°C). If you plan to read FP on the following day, seal the plates to prevent evaporation.

5.2.2 Stop Buffer A and SAH Detection Mixture Stability

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 (on ice or at 4°C) until they are used.

5.2.3 Stopping the Reaction

We have inhibited the activity of several histone and DNA methyltransferases by the addition of Stop Buffer A, by lowering the pH of the reaction and by quenching EDTA with MgCl₂. If the target MT enzyme requires >25 mM EDTA, additional MgCl₂ may be necessary to stop the reaction.

6.0 Troubleshooting

| Problem | Possible Causes and Solutions |
|---|---|
| Low selectivity | <p><i>Suboptimal antibody concentration</i></p> <ul style="list-style-type: none"> To achieve maximum sensitivity and assay window, the AMP²/GMP² Antibody concentration must be optimized for each starting SAM concentration. <p><i>SAM concentration out of range</i></p> <ul style="list-style-type: none"> Ensure that the starting SAM concentration is in the range of 0.1–50 μM. |
| No change in FP observed | <p><i>Low antibody/tracer activity</i></p> <ul style="list-style-type: none"> The tracer and antibody are stable for up to 6 freeze-thaw cycles. For frequent use, aliquot the antibody and tracer and store the aliquots at –20°C. Use a minimum of 20 μL aliquots. |
| High background signal or change in signal after incubation | <p><i>Interference from impurities</i></p> <ul style="list-style-type: none"> Nuclease contamination in the buffer can cause the assay window to collapse, causing a change in FP. We recommend using nuclease-free water and freshly prepared buffer for each assay. Some compounds may interfere with the detection mixture, causing a change in signal. Bovine serum albumin (BSA) at concentrations >1% interferes with the detection reagents. Detergents, such as Brij-35, can be substituted for BSA in the enzyme reaction to prevent nonspecific binding of enzymes and substrates to the plate. |

7.0 Appendix

7.1 Standard Curves

A standard curve (**Figure 5**) is required to convert mP values to product formation (SAH) for quantitative data analysis. Because the Transcreener® EPIGEN Methyltransferase Assay relies on a competitive binding reaction, the response is nonlinear, and therefore the signal is not directly proportional to reaction progress.

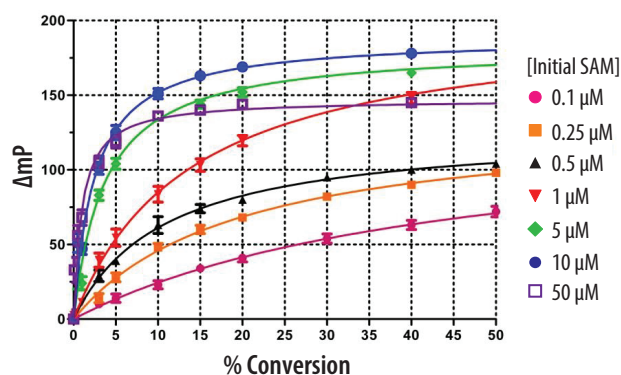
The wells for the standard curve should contain all MT reaction components except the enzyme and receive Stop Buffer A and Detection Mixture. The standard curve mimics an enzyme reaction (as SAM concentration decreases, SAH concentration increases); the total [SAM + SAH] concentration remains constant. The standard curve allows calculation of the concentration of SAH produced in the enzyme reaction and, therefore, the % SAM consumed (% conversion). In this example, a 12-point standard curve was prepared using the concentrations shown in **Table 1**. Commonly, 8- to 12-point standard curves are used.

Table 1. Concentrations of SAM/SAH to prepare a 12-point standard curve.

| % Conv. | SAH (nM) | SAM (nM) |
|---------|----------|----------|
| 100 | 100 | 0 |
| 60 | 60 | 40 |
| 40 | 40 | 60 |
| 30 | 30 | 70 |
| 20 | 20 | 80 |
| 15 | 15 | 85 |
| 10 | 10 | 90 |
| 7.5 | 7.5 | 92.5 |
| 5 | 5 | 95 |
| 3 | 3 | 97 |
| 1 | 1 | 99 |
| 0 | 0 | 100 |

Figure 5. Standard curve.

A) Sample data for standard curves starting at initial SAM concentrations of 0.1, 0.25, 0.5, 1, 5, 10, and 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 and a Z' value of 0.5 indicates robust assay performance for HTS applications. These criteria were achieved when less than 5% of the SAM was converted to SAH at initial SAM concentrations of 5 μM , 10 μM and 50 μM ; 10% conversion was required at initial SAM concentrations of 1 μM and 0.5 μM ; and 15% conversion and 30% conversion for 0.25 μM and 0.1 μM SAM, respectively.



Use the following equations to calculate the Z' factor:

$$\Delta mP = mP_{\text{initial [SAM]}} - mP_{\text{sample}}$$

$$Z' = 1 - \frac{[(3 \times SD_{\text{initial [SAM]}}) + (3 \times SD_{\text{sample}})]}{|(mP_{\text{initial [SAM]}}) - (mP_{\text{sample}})|}$$

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