# Neutrophil myeloperoxidase measurements in plasma, laminar tissue, and skin of horses given black walnut extract

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**Objective**—To compare measurements of myeloperoxidase (MPO) in plasma, laminar tissues, and skin obtained from control horses and horses given black walnut heartwood extract (BWHE).

**Animals**—22 healthy 5- to 15-year-old horses.

**Procedures**—Horses were randomly assigned to 4 groups as follows: a control group given water (n = 5) and 3 experimental groups given BWHE (17) via nasogastric intubation. Experimental groups consisted of 5, 6, and 6 horses that received BWHE and were euthanatized at 1.5, 3, and 12 hours after intubation, respectively. Control horses were euthanatized at 12 hours after intubation. Plasma samples were obtained hourly for all horses. Laminar tissue and skin from the middle region of the neck were harvested at the time of euthanasia. Plasma and tissue MPO concentrations were determined via an ELISA; tissue MPO activity was measured by use of specific immunologic extraction followed by enzymatic detection.

**Results**—Tissues and plasma of horses receiving BWHE contained significantly higher concentrations of MPO beginning at hour 3. Laminar tissue and skin from horses in experimental groups contained significantly higher MPO activity than tissues from control horses. Concentrations and activities of MPO in skin and laminar tissues were similar over time.

**Conclusions and Clinical Relevance**—In horses, BWHE administration causes increases in MPO concentration and activity in laminar tissue and skin and the time of increased MPO concentration correlates with emigration of WBCs from the vasculature. These findings support the hypothesis that activation of peripheral WBCs is an early step in the pathogenesis of acute laminitis. (*Am J Vet Res* 2007;68:81–86)

Acute laminitis occurs secondary to a variety of insults, including gastrointestinal diseases, pleuritis, retained placenta, carbohydrate overload, and exposure to shavings from black walnut trees.[1] Results of recent studies[2,3] indicate that peripheral WBCs become activated during the prodromal stage of acute laminitis induced by administration of an aqueous BWHE and leave the circulation. Concomitant with this leukopenic response are increases in leukocyte production of radical oxygen species, numbers of WBCs in laminar soft tissues, and expression of inflammatory mediators in the same tissues.[3-5] These early inflammatory events precede the development of morphologic changes in the digit, including interstitial edema, epidermal cell necrosis, and separation and loss of the basement membrane, that characterize laminitis.[6-10] Although the laminar tissue is a highly specialized tissue able to withstand large biomechanical forces, it remains part of the common integument, which includes the skin, ergots, and the chestnuts. Consequently, a broader examination of integument in horses given laminitis-inducing substances may provide additional insight into the pathogenesis of the disease.

**Abbreviations**

| | |
|---|---|
| BWHE | Black walnut heartwood extract |
| MPO | Myeloperoxidase |
| SIEFED | Specific immunologic extraction followed by enzymatic detection |
| MMP | Matrix metalloproteinase |

Normal equine integument contains few neutrophils,[5,11] and these cells may be difficult to detect under pathologic conditions because they rapidly undergo apoptotic degeneration.[10] However, the presence of these cells in tissues may be documented by monitoring the presence of MPO, a leukocyte-specific lysosomal enzyme.[12] Stimulation of neutrophils leads to their activation and release of MPO, which catalyzes the production of reactive oxygen species that can cause local tissue damage, including disruption of the laminar

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basement membrane.[13] Although MPO concentrations have been used as a marker of neutrophil presence in other equine tissues and plasma,[12,14-17] concentrations of MPO in equine laminar tissue and skin have not, to our knowledge, been previously reported.

The purpose of the study reported here was to compare results obtained with 2 new laboratory techniques designed to monitor changes in MPO concentration or activity. These techniques were used to characterize temporal changes in MPO in plasma, laminar tissues, and skin obtained from control horses and horses given BWHE. Hypotheses of this study were that administration of BWHE results in increased concentration and activity of MPO in plasma, laminae, and skin, compared with values from control horses; that tissue concentrations of MPO in horses given BWHE increase after the onset of leukopenia; and that concentrations of MPO in the skin correlate with values in the laminar tissues in individual horses. We elected to study the effects of BWHE to be able to directly compare our results with the results of recently published studies[3,4,18] that have implicated activation of peripheral WBCs in pathophysiologic causes of acute laminitis.

## Materials and Methods

**Animals**—Healthy horses ranging in age from 5 to 15 years were used in this study. All horses included were free of existing lameness and lacked clinical evidence of systemic inflammatory disease. No radiographic evidence of preexisting laminitis was present on survey lateral and dorsopalmar radiographic views of the forelimb digits. A 12-gauge catheter was placed in the left jugular vein of each horse for serial blood sample collection, and the middle region of the neck was clipped prior to the start of the study. The University of Georgia Animal Care and Use Committee approved the study.

**BWHE preparation**—The BWHE was prepared as described previously.[19] Briefly, 1 kg of black walnut heartwood shavings was agitated in 7 L of water at room temperature (approx 22°C) for 24 hours. An aqueous filtrate was then obtained by filtering the solution through cheesecloth. Six liters of the resulting BWHE was administered by nasogastric tube to horses.

**Experimental protocol**—Horses were randomly assigned to 1 of the 4 following groups: control, 1.5-hour BWHE, 3-hour BWHE, and 12-hour BWHE. Horses in the control group (5 horses) received 6 L of water via nasogastric intubation and served as controls; all horses in this group were euthanatized after the 12-hour sample collection. Horses in the 1.5-hour BWHE group (5 horses) received BWHE via nasogastric intubation and were euthanatized at 1.5 hours after intubation. Horses in the 3-hour BWHE group (6 horses) received BWHE via nasogastric intubation and were euthanatized at the onset of leukopenia (approx 3 hours after administration of BWHE and defined as a ≥ 30% decrease from time 0 peripheral WBC counts). Horses in the 12-hour BWHE group (6 horses) received BWHE via nasogastric intubation and were euthanatized at the onset of Obel grade 1 laminitis (clinical signs consisting of weight shifting and bounding digital pulses without evidence of lameness at a walk) or at 12 hours after intubation, if signs of Obel grade 1 laminitis had not developed by that time.

Each horse was evaluated prior to intubation and every hour thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade 1 laminitis. For horses in the control and 12-hour BWHE groups, blood samples were obtained via the jugular catheter at 0, 1, 2, 3, 4, 6, 8, 10, and 12 hours; blood samples were obtained at 0 and 1.5 hours from horses in the 1.5-hour BWHE group and at 0, 1.5, and 3 hours from horses in the 3-hour BWHE group. Blood samples were aliquoted into duplicate vacuum-evacuated tubes containing EDTA.[a] One sample was used for determination of the peripheral WBC count, whereas the other sample was immediately centrifuged at 400 × *g*. Plasma from the latter tube was frozen at −80°C until assayed for MPO concentration. All horses were euthanatized with a penetrating captive bolt, in compliance with guidelines outlined in the 2000 Report of the AVMA Panel on Euthanasia.

**Collection of laminar tissue and skin**—Both forelimbs were disarticulated at the level of the metacarpophalangeal joint, and hooves were cut into sections with a band saw. During the procedure, thermal damage was minimized by constant irrigation of the tissue with ice-cold physiologic salt solution containing the following: 118mM NaCl, 24mM $NaHCO_3$, 1mM $MgSO_4$, 0.435mM $NaH_2PO_4$, 5.56mM glucose, 1.8mM $CaCl_2$, and 4mM KCl. Two full-thickness segments from each forelimb foot were then placed in the ice-cold physiologic salt solution. The hard, keratinized portion of the hoof and distal phalanx were removed, and specimens of laminar tissue were removed by sharp dissection. Concurrently, a second investigator removed full-thickness, 6 cm × 6-cm sections of skin from the middle region of the neck. These specimens were also placed in the ice-cold physiologic salt solution. Laminar and skin specimens were rapidly frozen in liquid nitrogen and stored at −80°C.

Frozen tissue specimens were homogenized with a polytron tissue homogenizer[b] in Western lysis buffer containing 50mM HEPES, 150mM NaCl, 1% Triton X-100, 1mM EGTA, 6mM sodium deoxycholate, 1mM $Na_3VO_4$, 1mM NaF, 1mM phenyl-methane-sulfonyl fluoride, aprotinin (20 mg/mL), and leupeptin (20 mg/mL). After homogenization, samples were sonicated and centrifuged at 14,000 × *g* for 15 minutes at 4°C. The supernatant was aliquoted and frozen at −80°C.

**Sample preparation for ELISA**—Immediately prior to performing the ELISA, plasma and laminar supernatants were thawed and diluted 1:40 in a dilution buffer prepared with 20mM PBS solution (pH 7.4) containing bovine serum albumin (5 g/L) and 0.1% Tween 20. Skin supernatants were thawed and diluted 1:100 or 1:1,000 in the same dilution buffer.

**ELISA for measurement of MPO concentration**—Myeloperoxidase was measured with a specific sandwich ELISA[8] in which the primary antibody (rabbit anti-MPO

IgG) was coated onto microplate[c] wells. For standard preparation, MPO was extracted from equine neutrophils isolated from whole blood by sedimentation on density gradient followed by ion and gel filtration chromatography.[16] Equine MPO standards (ranging from 0.78 to 50 ng/mL) and samples containing MPO (100 µL) were added to the microplate and incubated overnight at 4°C with the primary antibody. After washing with saline (0.9% NaCl) solution containing 0.1% Tween 20, immobilized antibody-antigen complexes were incubated for 2 hours at 37°C with the secondary antibody (guinea pig anti-MPO IgG). After washing, a third antibody, produced in goats against guinea pig IgG and labelled with alkaline phosphatase,[d] was added to recognize the sandwich complex (ie, primary antibody–MPO–secondary antibody). After washing, phosphatase activity was detected by incubation (30 minutes, 37°C, in the dark) with the substrate paranitrophenyl phosphate[d] (2.7mM) in DEA buffer[d] (9.7% diethanolamine, 0.02% $NaN_3$, 0.01% $MgCl_2$, pH 9.8). The reaction was stopped with 2.5M NaOH, and the absorbance (405 nm) was read with a plate reader.[e] Control (blank) and dilutions of MPO standards and samples were made with the dilution buffer, and each sample was run in duplicate. The absorbance value was directly proportional to the quantity of the sandwich complex and, therefore, to the concentration of MPO in the sample.

**SIEFED assay for measurement of MPO activity**—The SIEFED method, developed specifically for measurement of equine MPO, is used to measure MPO activity in biological fluids.[20] The SIEFED method consists of capture of MPO from a tissue homogenate by immobilized (microplate-coated) specific antibodies, followed by elimination of the homogenate by washings and an in situ detection of the enzyme activity with a sensitive fluorogenic substrate (Amplex red) and a nitrite-based amplifier system. The primary antibody (rabbit anti-MPO IgG) was coated onto black microplate wells.[c] The MPO was extracted from equine neutrophils isolated from whole blood by sedimentation on density gradient, followed by ion and gel filtration chromatography and used for MPO standards.[16] Equine MPO standards (ranging from 0.25 to 6.4 mU/mL) and nondiluted samples containing MPO (200 µL) were added to the microplate and incubated for 2 hours at 37°C. After 3 washings, the peroxidase activity of MPO was detected by adding 100 µL of 40µM Amplex red[f] (10-acetyl-3, 7-dihydroxyphenoxazine), freshly prepared in phosphate buffer (50mM) at pH 7.5 containing 10µM $H_2O_2$ and 10mM nitrite. Fluorescence was measured with a fluorescence scanning instrument[g] at the excitation and emission wavelengths of 544 and 590 nm, respectively. Controls (blanks) were prepared with the dilution buffer, and each sample was run in duplicate. The fluorescence value was directly proportional to the quantity of active MPO in the sample.

**Statistical analysis**—Statistical analyses were performed with commercially available software.[h] Concentrations and activities of MPO in laminar tissue were compared with those in skin by linear regression. Similar methods were used to compare values for each tissue obtained with the 2 methods of MPO detection, SIEFED and ELISA. Plasma and tissue MPO data were logarithmically transformed (natural logarithm) for normal distribution of data before analysis. Data for each time point for skin and laminar specimens were compared for ELISA and SIEFED results by use of a 1-way ANOVA with Bonferroni posttest and unpaired *t* test on transformed data. An unpaired *t* test with the Welch correction for unequal variances was used to compare plasma concentrations for multiple time points with logarithmically ltransformed (natural logarithm) data. For all comparisons, a value of $P < 0.05$ was considered significant.

## Results

**Plasma MPO concentrations determined by ELISA**—Mean MPO concentrations in plasma samples obtained at time 0 did not differ significantly ($P = 0.74$) between the control and BWHE groups. In contrast, plasma concentrations of MPO in the BWHE group were significantly greater than those in the control group at 1, 2, 3, 4, 6, and 8 hours. Plasma concentrations of MPO within the control group were significantly lower than time 0 values at time points 1, 2, and 3 (**Figure 1**).

**Laminar tissue MPO concentrations determined by ELISA**—Concentrations of MPO in laminar specimens from the 3- and 12-hour BWHE groups were significantly greater than those of the control group (**Figure 2**). Concentrations of MPO in laminar tissues of the 3- and 12-hour BWHE groups were not significantly different.

**Skin MPO concentrations determined by ELISA**—Concentrations of MPO in skin specimens from the 3- and 12-hour BWHE groups were significantly greater than values for the control and 1.5-hour BWHE groups. No significant differences were found in MPO concentrations between the control and 1.5-hour BWHE groups or between the 3- and 12-hour BWHE groups.

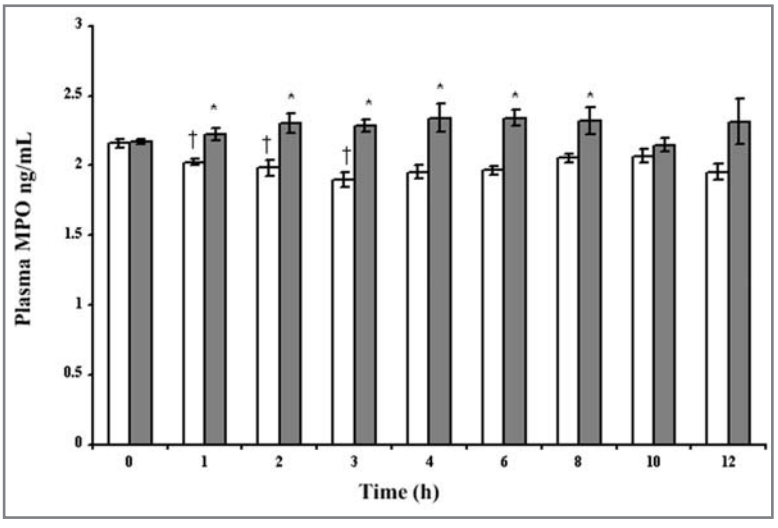


Figure 1—Mean ± SEM log concentration of MPO in plasma samples from 5 control horses (white bars) and 17 BWHE-treated horses (gray bars) for described time points. *Significantly ($P < 0.05$) higher than time 0 values. †Significantly ($P < 0.05$) lower than time 0 values.

Mean MPO concentrations in laminar and skin specimens obtained from horses in the control group were not significantly different (Figure 2). Mean MPO concentrations in skin and laminar tissues of horses 1.5 hours after administration of BWHE were not significantly different from values for control horses (**Table 1**).

**Laminar tissue activity determined by SIEFED**—Mean MPO activities in laminar tissue and skin specimens were determined (**Figure 3**). Activities of MPO in laminar specimens from the 1.5-, 3-, and 12-hour BWHE groups were significantly greater than values for the control group (Table 1). No significant differences were found in MPO activities between the 3- and 12-hour BWHE groups.

**Skin activity determined by SIEFED**—Activities of MPO in skin specimens from the 1.5-, 3-, and 12-hour BWHE groups were significantly greater than those from the control group. No significant differences were found in MPO activities between the 3- and 12-hour BWHE groups.

**Correlations between ELISA and SIEFED data**—Positive linear correlations were identified for MPO concentrations between laminar tissue and skin obtained by use of ELISA ($R^2 = 0.51$) and SIEFED ($R^2 = 0.70$). A comparison of individual MPO values obtained with ELISA and SIEFED yielded positive linear correlations for laminar tissue ($R^2 = 0.51$) and skin ($R^2 = 0.64$).

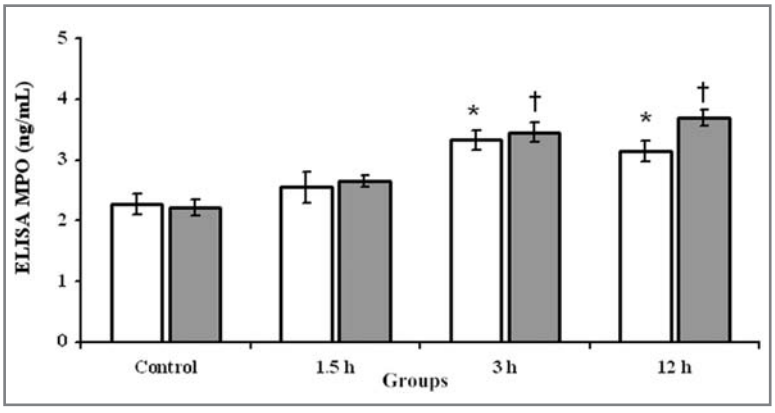


Figure 2—Mean ± SEM log concentration of MPO in laminar tissue (white bars) and skin (gray bars) from control and BWHE-treated horses for described time points determined by an ELISA. *Significantly ($P < 0.05$) higher than control values in laminar tissue. †Significantly ($P < 0.05$) higher than control values in skin.

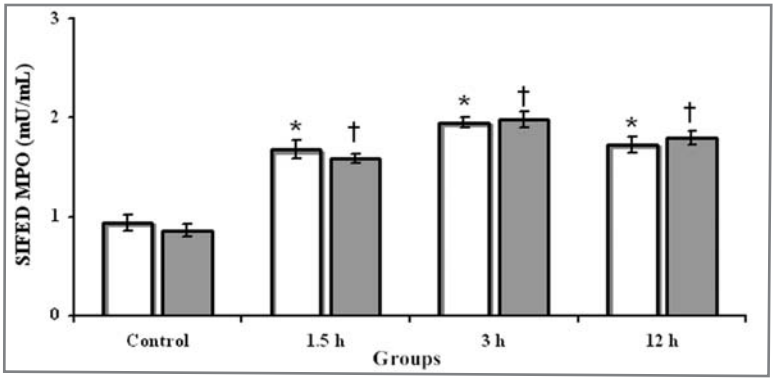


Figure 3—Mean ± SEM log activity of MPO in laminar tissue (white bars) and skin (gray bars) from control and BWHE-treated horses for described time points determined by an SIEFED. *See* Figure 2 for remainder of key.

## Discussion

Results of our study provide convincing evidence for systemic activation of neutrophils and their emigration into the integument in horses given BWHE. In our study, plasma concentrations of MPO at time 0 in both groups of horses were within normal reference limits for horses, suggesting that little intravascular release of neutrophil granular contents occurs under basal conditions. However, plasma concentrations of MPO increased by 1 hour after administration of BWHE and remained significantly increased throughout the 8-hour period. This early increase in circulating concentrations of MPO is consistent with intravascular activation and degranulation of neutrophils and coincides with the development of leukopenia as activated cells leave the circulation. The sustained increase in MPO concentration in plasma is indicative of continued intravascular activation of WBCs and coincides with increased production of reactive oxygen species by WBCs after horses received BWHE.[3,5] To be fair, however, it cannot be determined whether the increased plasma concentrations of MPO were solely caused by intravascular release of the enzyme or if some MPO released in adjacent tissues reentered the circulation. For example, recent evidence suggests that the administration of BWHE damages colonic mucosal epithelium, thus compromising the mucosal barrier.[21] This damaging effect of BWHE may be attributable to upregulation of proinflammatory mediators and cytokines that in turn could lead to neutrophil activation and release of MPO in plasma.

Concentrations and activities of MPO in skin and laminar tissue of control horses were low, consistent with recent reports[5,11]

Table 1—Mean ± SEM values for tissue MPO concentrations and MPO activities in laminar tissue and skin.

| | ELISA | | SIEFED | |
|---|---|---|---|---|
| **Group** | **Lamina (ng/mL)** | **Skin (ng/mL)** | **Lamina (mU/mL)** | **Skin (mU/mL)** |
| Control (n = 5) | 236.3 ± 61.73 | 196.4 ± 51.5 | 9.4 ± 1.6 | 7.7 ± 1.1 |
| 1.5-h BWHE (5) | 651.4 ± 335.6 | 516.4 ± 153.9 | 53.0 ± 12.7* | 39.7 ± 4.8* |
| 3-h BWHE (6) | 3,342.9 ± 1,726.8* | 4,122.5 ± 1,559.0* | 92.7 ± 11.1* | 107.3 ± 25.2* |
| 12-h BWHE (6) | 2,455.5 ± 1,504.1* | 6,168.5 ± 1,913.2* | 60.0 ± 14.4* | 68.7 ± 14.3* |

*Significantly ($P < 0.05$) different from control group.

that relatively few neutrophils are present in these tissues in healthy horses. In 1 report,[5] ≤ 1 $CD13^+$ cell (ie, neutrophils or monocytes)/40× field in laminae from 5 healthy horses were present and 0 to 3 $CD13^+$ cells were present in skin from 4 of the 5 horses. Consequently, overlap in MPO values obtained for laminae and skin of the control horses is not unexpected. By 3 hours after administration of BWHE, however, MPO concentrations and activities were significantly increased in laminae and skin and remained increased at the 12-hour time point. Collectively, these results provide supportive evidence for the recent observation that the number of neutrophils in laminar tissue and skin increases as an early response to BWHE administration.[5] Taken together, those findings and our results indicate that the inflammatory processes initiated by intragastric administration of BWHE are not restricted to the laminar soft tissues, but occur in the integument in general.

Degradation of the laminar basement membrane is a histologic hallmark of acute laminitis in horses. An increase in the local activity of MMPs has been theorized to be the cause of this basement membrane disruption.[4,22,23] The MMPs comprise a family of enzymes that degrade and remodel various components of the extracellular matrix. Several studies[18,22,23] have documented increases in MMP-2 and -9 in laminar tissue in acute laminitis. Although MMP-2 is produced by local cellular components, MMP-9 is a product of activated neutrophils.

Results of our study provide additional support for the hypothesis that activated neutrophils play a role in initiating the local inflammatory responses that occur in the laminar soft tissues during the development of acute laminitis. Neutrophils are activated by endogenous processes that ultimately induce their extravasation and degranulation with the release of MPO. In our study, increases in tissue MPO concentrations and activities were accompanied by increases in plasma concentrations of MPO, suggesting that intragastric administration of BWHE results in systemic activation of neutrophils. Increases in plasma MPO concentrations are found in horses with strangulating large intestinal obstructions and decreased numbers of circulating WBCs, suggesting that the increased circulating MPO concentrations originated from neutrophils that had become activated within the circulation.[15] These findings also are consistent with the recent report[24] that horses with strangulating intestinal lesions have activated neutrophils in circulation.

Although the primary purpose of our study was to monitor changes in MPO concentration or activity as markers for the presence of neutrophils, a direct role of MPO in the pathogenesis of acute laminitis should not be overlooked. Myeloperoxidase generates a variety of reactive oxygen species, increases the oxidative potential of hydrogen peroxide, and is strongly associated with the development of endothelial cell dysfunction in laboratory animals and humans.[25-28] For example, MPO activity is linked with a reduction in the bioavailability of nitric oxide, inhibition of nitric oxide synthase activity, and the development of atherosclerotic cardiovascular disease in people.[25,29] Thus, it is likely that increased local activity of MPO plays a prominent role in the development of laminar microvascular dysfunction in horses developing acute laminitis.

To our knowledge, this is the first report on MPO concentrations and activities in horses with experimentally induced laminitis and, therefore, serves as another step in the ongoing effort to elucidate the pathophysiologic causes of acute laminitis. Because the increases in MPO concentration and activity occurred in the skin and laminar tissue, these findings provide support for future studies designed to determine whether MPO concentrations and activities in skin specimens can be used as biochemical markers of the inflammatory processes involved in the pathogenesis of acute laminitis. With this approach, it may be possible to monitor changes in MPO concentration and activity in serial skin biopsies as indicators of the morphologic changes in laminar tissue. The ease with which skin specimens can be obtained, compared with the collection of laminar tissue, may facilitate identification of horses at increased risk of developing acute laminitis and assessment of the efficacy of new treatment modalities designed to minimize activation and extravasation of WBCs.

a. Becton-Dickinson, Franklin Lakes, NJ.
b. Glen Mills Inc, Clifton, NJ.
c. Microplate wells, Thermo Labsystems, Waltham, Mass.
d. Sigma Chemical Co, St Louis, Mo.
e. Multiscan Ascent plate reader, Thermo Labsystems, Waltham, Mass.
f. Molecular probes, Eugene, Ore.
g. Fluoroscan Ascent instrument, Thermo Labsystems, Waltham, Mass.
h. GraphPad Prism, version 3.03, GraphPad Software, San Diego, Calif.

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