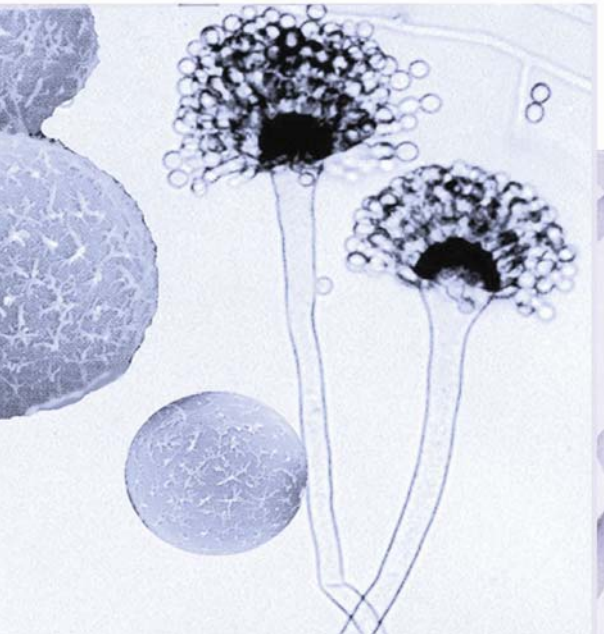
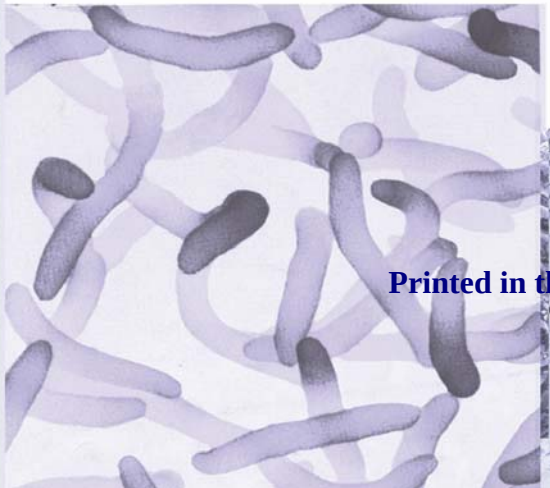
Biolog Redox Dye Mixes

for Enumerating Mammalian Cells
in Proliferation and Chemosensitivity Assays

PRODUCT DESCRIPTIONS AND INSTRUCTIONS FOR USE
Biolog Redox Dye Mix MA, (6x), Cat. # 74351
Biolog Redox Dye Mix MB, (6x), Cat. # 74352





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I. Introduction

a. Overview

Biolog Redox Dye Mixes are easy-to-use colorimetric reagents for determining the number of viable cells, making them ideal for applications in cell proliferation and chemosensitivity or cytotoxicity assays. Both mixes contain a tetrazolium-based reagent that can be reduced to a soluble purple formazan product by live cells in a variety of tissue culture media. The amount of formazan is measured spectrophotometrically at 590 nm directly in microwell assay plates without additional processing. The quantity of formazan product is directly proportional to the number of living and respiring cells.

b. Advantages

The Biolog Redox Dye Mix Assay has all the advantages of using a water soluble tetrazolium-based assay:

- **Proven Technology:** A sizeable published literature documents the successful use of colorimetric tetrazolium assays in cell proliferation and chemosensitivity studies [1-17].
- **Simple Protocol:** Add the dye mix to cells, incubate and measure absorbance.
- **Easy to Use:** Assays are performed in standard 96- and 384-well microplates without additional cell washing or harvesting steps.
- **Fast Results:** Sufficient color forms in as little as 30 minutes. The formazan product is soluble in tissue culture medium and, unlike MTT which precipitates inside cells, does not require solubilization steps.
- **Avoids Hazards:** No volatile organic solvents are required to solubilize the formazan product as in MTT assays. No scintillation cocktail or radioactive waste disposal is required as in radioactive thymidine labeling assays for newly synthesized DNA.
- **Flexible Format:** Color development can be monitored visually or by instrument throughout the assay, allowing for either kinetic or endpoint measurements. Assay sensitivity can be enhanced simply by extending the incubation time. In contrast, cell enumeration with MTT is performed as a fixed-time endpoint assay only.

In addition, the Biolog Redox Dye Mix Assay has these unique advantages:

- **Purple formazan color:** With maximal formazan absorbance at 590 nm (purple) there is less interference from colored chemicals (yellow, orange, brown) that are common in chemosensitivity assays and which typically absorb between 400 nm and 500 nm (Fig. 4).
- **Low Background:** Biolog Redox Dye Mixes have low abiotic reduction. At 37°C in RPMI medium without phenol red containing 5% serum, Biolog Redox Dye Mix MA has an A_{590} value of 0.046 ± 0.001 after 1 hour, and MB has an A_{590} value of 0.064 ± 0.004 after 6 hours. Since the A_{590} of the medium lacking dye is 0.036 ± .001, these low values enable much higher signal to background ratios and sensitivity. Other water soluble tetrazolium chemistries exhibit high background due to abiotic reduction in serum-containing media.
- **High Sensitivity:** Efficient dye reduction coupled to a molar extinction coefficient of 12,955 cm^{-1} M^{-1} for the purple formazan makes it possible to detect less than 300 cells/well for many cell lines in 96-well plates (Table 1; Figs. 1, 2, 3, and inset).
- **High Dynamic Range:** Linear enumeration of cells up to 160,000 per well can be achieved.
- **Broad Applicability:** Biolog offers two Redox Dye Mixes for use with cells. Both have been demonstrated to work with a wide range of cell types that include: liver (HepG2 and

HepG2/C3A), colon (COLO205), lung (A549), glioma (C6), ovary (CHO-K1), kidney (HEK293) prostate (PC-3), breast (MCF-7), neuroblastoma (NG108-15), hybridoma (413-15D12), retina epithelial (ARPE-19) blood cells (CCRF-CEM and HL-60) and fibroblasts (IMR-90) (Table 1).

- **Serum Independent Color:** Because Biolog Redox Dye Mixes have a stable absorption spectrum over a wide range of serum concentrations, absorbance values can be compared from cells incubated in media containing 0% to 20% serum. Other dyes can exhibit serum-dependent spectral shifts upon formazan binding to serum proteins.
- **Use with Biolog Phenotype MicroArrays™:** Only Biolog Redox Dye Mixes can be used with Biolog Phenotype MicroArrays to measure energy-production of cells stimulated by specific oxidizable substrates such as glucose and pyruvate.

II. Product Description

Two Dye Mixes are available for use with a wide range of cell types (Table 1 and 2). Most often, the tetrazolium in Biolog Redox Dye Mix MB will be reduced by cells. Biolog Redox Dye Mix MB is also much better for blood cells and some other cell lines (e.g. HEK293, CHO-K1, 413-15D12, IMR-90). It is recommended that both Biolog Redox Dye Mix MA and MB be tested on any new cell line to see which is preferable. Both Biolog Redox Dye Mixes allow for the counting of viable cells based on the linear increase in A_{590} of the formazan with cell number (Figs. 1, 2 and 3).

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- **Concentration:** The Dye Mixes are sold as 6x concentrates. When used and diluted to the recommended concentration, the final concentration of tetrazolium in the assay is 500 µM.

- **Size:** 20 ml, typically enough for 1,000 wells of microplate assays with 100 µl of culture per well, or 2,000 wells of microplate assays with 50 µl of culture per well.

- **Intended Use:** Biolog Redox Dye Mix MA and MB are intended for Laboratory Use Only.

- **Storage:** Working solutions of Biolog Redox Dye Mixes are stable for months at 4°C when protected from light. Long-term storage is best at -20°C. See the expiration date on the product label.

- **Linear Readout:** Biolog Redox Dye Mixes MA and MB allow for the linear enumeration of cell lines (Figs. 1, 2, and 3).

- **Cell Lines:** The two Biolog Redox Dye Mixes MA and MB allow for optimizing assay conditions for numerous cell lines (Table 1and 2).

TABLE 1. BIOLOG REDOX DYE MIX MA OR MB PREFERENCE BY CELL LINE[1]

Cell Line	Organism and Cell Type	Preferred Dye Mix	Rate of Dye Reduction[3]	Number of Cells Detected in Wells			
				Lowest[4]	Time (h)	Highest[5]	Time (h)
413-15D12	Mouse/Rat Hybridoma	MB	S	2,500	6	80,000	2
A549	Human Lung Carcinoma	MB[2]	F	625	0.75	40,000	0.75
ARPE-19	Human Retina Epithelial	MB[2]	F	156	1	40,000	1
C3A	Human Liver Carcinoma	MA[2]	M	625	0.5	160,000	1
C6	Rat Glioma	MA[2]	M	625	0.5	160,000	1
CCRF-CEM	Human Leukemia Lymphoblast	MB	S	5,000	2	160,000	6
CHO-K1	Hamster Ovary	MB	M	625	0.75	160,000	2
COLO205	Human Colon Carcinoma	MB[2]	F	312	0.75	80,000	1
HEK293	Human Embryonic Kidney	MB	M	625	1	160,000	1
HEPG2	Human Liver Carcinoma	MA[2]	M	156	0.75	80,000	1
HL-60	Human Leukemia Promyeloblast	MB	S	625	1	160,000	1
IMR-90	Human Lung Fibroblast	MB	M	78	1	40,000	1
MCF-7	Human Breast Carcinoma	MB[2]	M	312	0.75	80,000	1
NG108-15	Mouse/Rat Neuroblastoma	MB	M	312	0.75	80,000	1
PC-3	Human Prostate Carcinoma	MA[2]	M	625	0.75	80,000	1

[1]Biolog Redox Dye Mix MA or MB (10 µl) was added to wells containing known number of cells in 50 µl RPMI 1640 lacking phenol red + 5% fetal bovine serum + 1x Pen/Strep. The amount of tetrazolium reduced to a colored formazan at 37°C was measured by A_{590} every 15 minutes for an hour, then every hour for 6 hours.

[2]Other Biolog Redox Dye Mix works comparably well.

[3]S = Slow, M = Moderate, F = Fast

[4]Each cell line was seeded in a Biolog half-area 96-well microplate. Cells were 2-fold serial diluted from 160,000 to 156 cells per well in 50 µl RPMI without phenol red, supplemented with 5% fetal bovine serum and 1x Pen/Strep. Each assay was allowed to equilibrate for 1 h at 37°C in a humidified, 95% Air-5% CO_2 atmosphere before 10 µl of Biolog Redox Dye Mix MA or MB were added. The mean and SD of 4 replicates (n = 4) were calculated at each cell density. Significant levels of dye reduction was determined by the Student's T test ($p < 0.01$), which was used to find the lowest cell number detected in the wells at the corresponding assay time.

[5] Denotes the highest cell density at the indicated longest assay time to remain linearly proportional to A_{590} values, which was determined by linear regression analysis (R^2 was greater than 0.97).

TABLE 2. BIOLOG REDOX DYE MIX MA OR MB PREFERENCE[1]

Preferred Dye Mix	Rate of Dye Reduction[3]	Cell Line	Organism and Cell Type	Number of Cells Detected in Wells			
				Lowest[4]	Time (h)	Highest[5]	Time (h)
MA[2]	M	C6	Rat Glioma	625	0.5	160,000	1
MA[2]	M	PC-3	Human Prostate Carcinoma	625	0.75	80,000	1
MA[2]	M	HepG2/C3A	Human Liver Carcinoma	625	0.5	160,000	1
MA[2]	M	HepG2	Human Liver Carcinoma	156	0.75	80,000	1
MB[2]	F	A549	Human Lung Carcinoma	625	0.75	40,000	0.75
MB[2]	F	COLO205	Human Colon Carcinoma	312	0.75	80,000	1
MB[2]	F	ARPE-19	Human Retina Epithelial	156	1	40,000	1
MB	M	CHO-K1	Hamster Ovary	625	0.75	160,000	2
MB	M	HEK293	Human Embryonic Kidney	625	1	160,000	1
MB[2]	M	MCF-7	Human Breast Carcinoma	312	0.75	80,000	1
MB	M	NG108-15	Mouse/Rat Neuroblastoma	312	0.75	80,000	1
MB	M	IMR-90	Human Lung Fibroblast	78	1	40,000	1
MB	S	CCRF-CEM	Human Leukemia Lymphoblast	5,000	2	160,000	6
MB	S	413-15D12	Mouse/Rat Hybridoma	2,500	6	80,000	2
MB	S	HL-60	Human Leukemia Promyeloblast	625	1	160,000	1

[1, 2, 3, 4, and 5] see Table 1 for information.

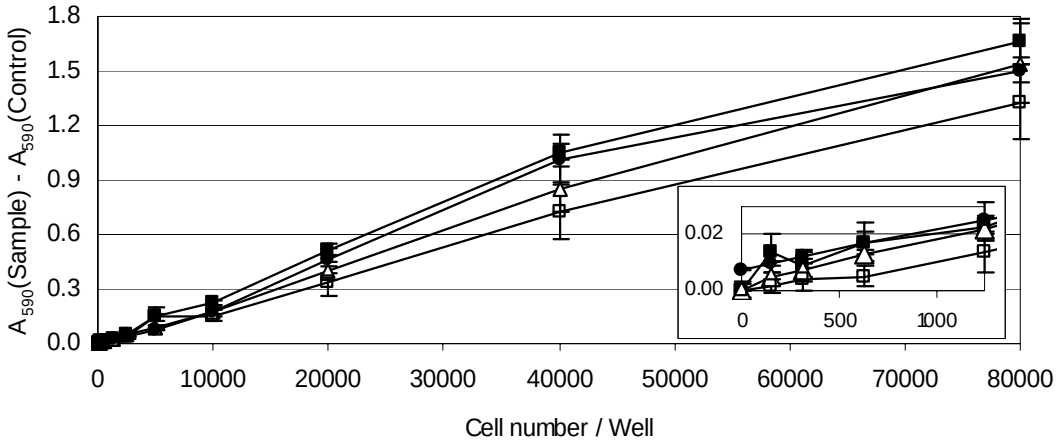

FIG. 1 Biolog Redox Dye Mix MA after 1 h at 37°C produces linear increase of A_{590} with increasing number of HEPG2/C3A (*filled square*), PC-3 (*filled circle*), C6 (*open triangle*), and HepG2 (*open square*) cells with R^2 linearity correlations of 0.995, 0.977, 0.998, and 0.996, respectively. Inset displays sensitivity at cell numbers less than 1,250 per well. All cells were dispensed at indicated cell numbers in RPMI without phenol red containing 5% fetal bovine serum and 1x Pen/Strep and incubated for 2 h before dye was added. All A_{590} values were corrected by subtracting the reference A_{590} value 0.046 which is the negative control well containing medium and dye mix, but no cells. Values represent the mean ± SD of four replicates (*n = 4*).

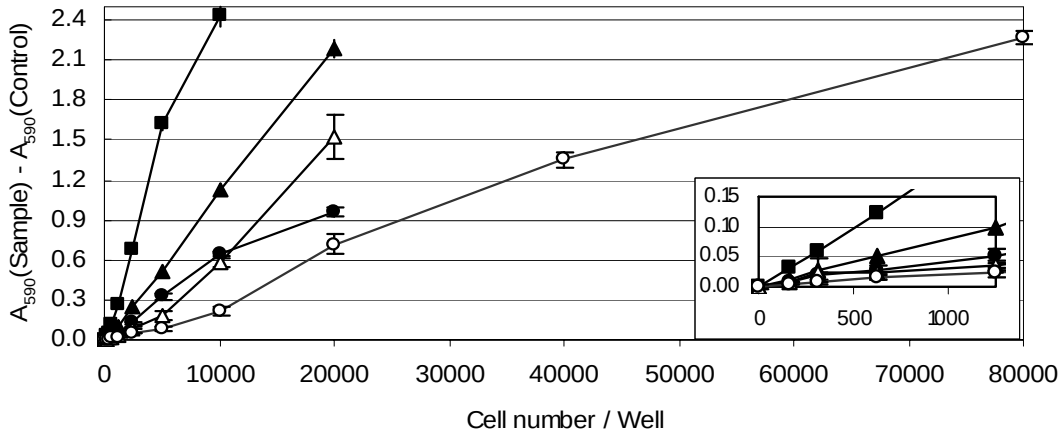

FIG. 2 Biolog Redox Dye Mix MB after 1 h at 37°C produces linear increase of A_{590} with increasing number of ARPE-19 (*filled square*), MCF-7 (*filled triangle*), A549 (*open triangle*), IMR-90 (*filled circle*), and COLO205 (*open circle*) cells with R^2 linearity correlations of 0.975, 0.999, 0.977, 0.973, and 0.988 respectively. Inset displays sensitivity at cell numbers less than 1,250 per well. All cells were dispensed at indicated cell numbers in RPMI without phenol red containing 5% fetal bovine serum and 1x Pen/Strep and incubated for 1 h before dye was added. All A_{590} values were corrected by subtracting the reference A_{590} value 0.049 which is the negative control well containing medium and dye mix, but no cells. Values represent the mean ± SD of four replicates (*n = 4*).

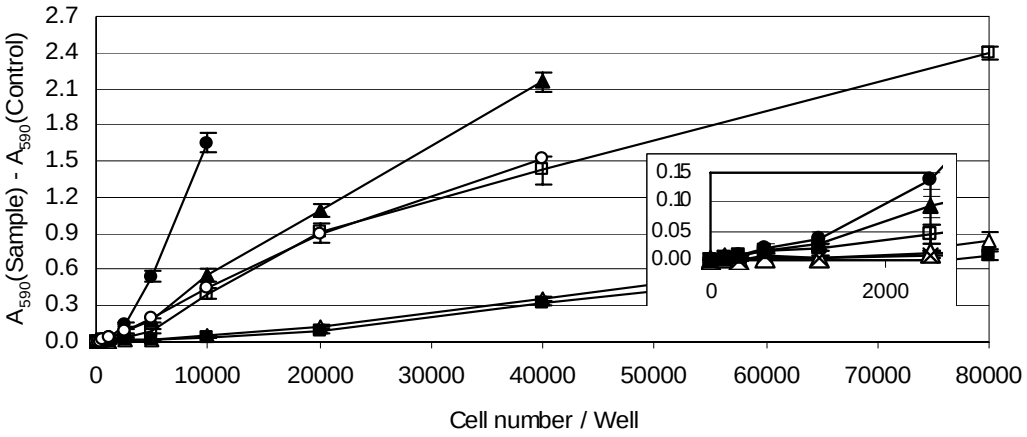

FIG. 3 Biolog Redox Dye Mix MB after 6 h at 37°C produces linear increase of A_{590} with increasing number of NG108-15 (*filled circle*) CHO-K1 (*filled triangle*), HL-60 (*open circle*), HEK293 (*open square*), 413-15D12 (*open triangle*), and CCRF-CEM (*filled square*) cells with R^2 values of 0.998, 0.978, 0.994, 0.984, and 0.979, respectively. Inset displays sensitivity at cell numbers less than 2,500 per well. All cells were dispensed at indicated cell numbers in RPMI without phenol red containing 5% fetal bovine serum and 1x Pen/Strep and incubated for 1 h before dye was added. All A_{590} values were corrected by subtracting the reference A_{590} value 0.064 which is the negative control well containing medium and dye mix, but no cells. Values represent the mean ± SD of four replicates (*n = 4*).

III. Protocols

a. Cell Counting

1. Harvest cells grown either in suspension or attached. Suspend the cells in growth medium (RPMI *etc*.) lacking phenol red, but containing 0 to 20 % serum and Pen/Strep. In duplicate, seed cells that have been sequentially diluted 2-fold to encompass a range of 160,000 to 0 cells/per well in volumes of 100, 50 or 25 µl for 96-well, 96-well half-area or 384-well plates, respectively.

2. Allow the cells to equilibrate for 1-2 hours in a humidified cell culture incubator with 95% Air- 5% CO_2 atmosphere at 37°C.

3. Prewarm the Biolog Redox Dye Mix MA and MB solutions to 37°C just prior to use. Different cell types display different optimal reduction of the tetrazolium in Dye Mix MA or MB so it is important to test both and determine which is best for specific cell lines.

4. For every 100 µl of culture volume, add 20 µl/well of either Biolog Redox Dye Mix MA or Biolog Redox Dye Mix MB.

   Note: The recommended concentrations of dye mix reagent has been optimized for a wide variety of cell lines cultured in 96-well plates containing 100 µl of medium per well. If different volumes of medium are used, adjust the volume of the dye mix reagent to maintain a ratio of 20 µl per 100 µl culture medium. This results in a final assay concentration of 500 µM tetrazolium dye.

5. Determine the formazan produced every 15 minutes for an hour, then every hour for 6 hours by evaluating the A_{590} or ($A_{590-750}$) with a microplate reader. Continue incubating the plate at 37°C in a humidified incubator while recording absorbance values for 1 to 6 hours (even overnight for cells that reduce the tetrazolium dye at slow rates).

   Important: *It is preferred that $A_{590-750}$ values are used to quantify the amount of formazan produced. Non-specific absorbance at 590 nm can be removed by subtracting A_{750} values. The purple formazan has negligible absorbance at 750 nm (Fig. 4). Alternatively, A_{590} of the sample can be subtracted from A_{590} of the negative control well containing medium and dye mix, but no cells provide the plates are uniform.*

6. Subtract the A_{590} value of well(s) containing medium and dye, but no cells, at each time point, from all A_{590} values. Plot the A_{590} as a function of cell number. Since A_{590} was taken every 15 minutes for an hour, then every hour for 6 hours, the kinetics of formazan production can be evaluated to ensure that endpoint readings are performed while the formazan production is linear with time. This protocol was used to construct the graphs shown in figures 1 through 3.

b. Cell Proliferation Assay

1. Use growth medium (RPMI *etc*.) lacking phenol red, seed 96-well plates, 96-well half-area plates, or 384-well plates with enough cells in 100, 50, or 25 µl, respectively, that will give an $A_{590-750}$ value significantly higher than background (well containing medium and dye, but not cells) when a cell counting assay is performed using either Biolog Redox Dye Mix MA or MB. The cell counting assay was described above in section III.a.

2. Prewarm the Biolog Redox Dye Mix solution just prior to use.

3. Dispense 20 µl of the dye mix solution to 100 µl of medium in each well of the assay plates (1 to 5 ratio of dye to medium).

4. Incubate plates at 37°C in a humidified incubator (under 95% Air-5% CO_2 for bicarbonate medium buffer systems) until color develops, typically 1 to 6 hours.

   Note: To measure the amount of soluble formazan produced by bioreduction of the dye, proceed immediately to Step 5. Alternatively, to measure the absorbance at a later time, add SDS to a final concentration of 2% (e.g., 25 µl of 10% SDS to 100 µl of cell culture) to each well to prevent further dye reduction. Store SDS-treated plates in a humid environment and protected from light at room temperature for up to 18 hours. Assay plates can also be stored at 4^{o}C overnight prior to taking readings.

5. Read and record the absorbance at 590 nm (A_{590}) or absorbance at 590 nm minus absorbance at 750 nm ($A_{590-750}$) using a microplate reader.

6. Proliferation over time can be determined from the amount of formazan produced which reflects the number of viable, respiring cells.

c. Chemosensitivity Assay

Cytotoxicity assays are performed essentially as cell proliferation assays but with cells exposed to appropriate concentrations of chemicals for appropriate periods of time prior to quantifying the number of the viable cells remaining by using the Biolog Redox Dye Mixes for cell enumeration. Refer to the following references for examples of typical chemosensitivity assays [1-16].

IV. General Considerations

a. Light Sensitivity

As with other tetrazolium compounds, Biolog Redox Dye Mixes are light sensitive and supplied in amber containers. Discoloration from orange to brown may occur if solutions are stored improperly. This discoloration can cause higher background A_{590} values, making it difficult to quantify the cell-mediated tetrazolium reduction. Dye Mixes that become discolored and have unacceptably high A_{590} background values, should be discarded.

b. Chemical Safety

Material Data Safety sheets for these products are available from Biolog. The toxicological properties of the Dye Mixes have not been thoroughly investigated, so caution should be used in handling them. Tetrazolium compounds are generally classified as irritants. Suitable precautions should be taken also in the disposal of this product.

c. Background Absorbance

A slight amount of 590 nm absorbance occurs due to abiotic reduction in serum-containing culture medium incubated with either Dye Mix solution. The composition and pH of the culture medium, type and concentration of serum, temperature, length of exposure to light and any chemicals added to the culture medium may contribute to formazan production and consequent increase in A_{590} background values.

For example, culture medium at elevated pH or with extended exposure to direct light may cause an accelerated non-cell mediated reduction of tetrazolium salts. Additionally, reducing substances including ascorbic acid and NADH, or sulfhydryl-containing compounds, such as L-cysteine, glutathione, coenzyme A, and dithiothreitol, and even strongly reducing sugars such as ribose and xylose can reduce tetrazolium salts nonenzymatically. Such chemicals may be ingredients of the culture medium or added to the cultures in chemosensitivity assays.

Background A_{590} values for Biolog Redox Dye Mix MA (at 1 h) and MB (at 1 and 6 h) at 37^{o}C in RPMI lacking phenol red but containing 5% fetal bovine serum and 1x Pen/Strep were 0.046 ± 0.001, 0.049 ± 0.001, and 0.064 ± 0.004 units, respectively. Unexpectedly high A_{590} values may indicate chemical interference from test compounds. This can be confirmed by measuring A_{590} values from control wells containing medium without cells at various concentrations of test compound.

If background A_{590} is significant using your experimental conditions, correct for it as follows. Prepare a triplicate set of control wells (without cells) containing the same volumes of culture medium and dye mix solution as in the experimental wells. Subtract the average A_{590} from these “no cell” control wells from all other A_{590} values to yield corrected absorbance values.

d. Optional Wavelengths to Read Absorbance

The reduced form of the Biolog Redox Dye Mixes absorbs maximally at 590 nm (Fig. 4) and this wavelength is recommended for determining the amount of formazan produced. If the microplate reader available does not have a 590 nm filter, wavelengths near 590 nm may be employed but this will decrease the assay sensitivity. Moreover, interference from colored chemicals added when performing chemosensitivity experiments may also result. Some chemicals tested in chemosensitivity assays are yellow, orange, or brown with high absorbance between 400 nm and 500 nm.

e. Dual Wavelength Reading

It is preferable to collect data in dual wavelength mode with a second reference wavelength at 750 nm. Use of this second reference wavelength eliminates background absorbance contributed by cell debris, fingerprints, plate scratches, etc. The most accurate and sensitive readings are obtained by using $A_{590-750}$ values.

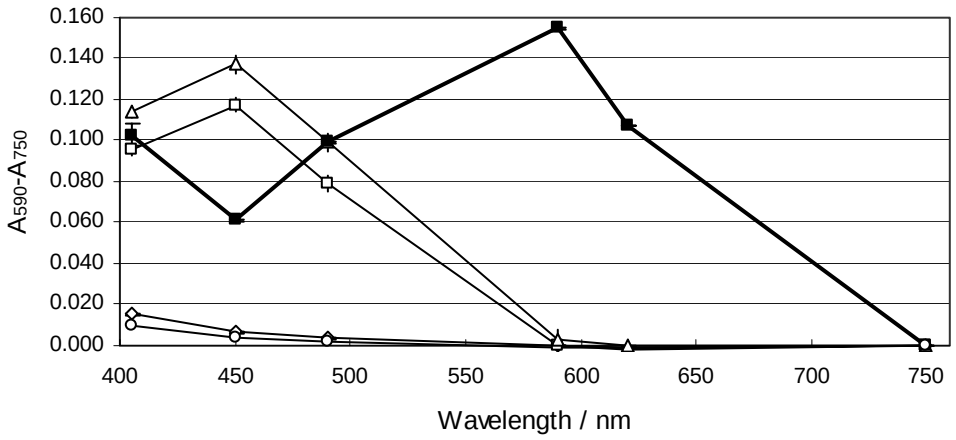

FIG. 4. ABSORBANCE spectrum of Biolog Redox Dye Mix MA, MB and the reduced soluble formazan. For unreduced dye, 10 µl of each Biolog Redox Dye Mixes were added to 50 µl of IF-M1, dispensed into a Biolog half-area 96-well microtiter plate lacking cells. The soluble formazan was measured from diluted dye mixes fully reduced with an excess of dithiolthreitol. The purple formazan (*filled square*), 1/20 of actual experimental values, has a maximal absorbance at 590 nm, while the absorbance of unreduced Dye Mix MA (*open square*) and MB (*open triangle*) is not detected. IF-M1 (*open diamond*) without dye has the same, almost negligible absorbance spectrum as water (*open circle*). The averaged background absorbance value of 0.043 at 750 nm was subtracted from all wavelength values. Error bars represent one standard deviation from eight independent readings (*n = 8*).

f. Blood and Hybridoma Cells Assays

Blood and hybridoma cells produce less formazan than other cell types (Fig. 3). To achieve significant absorbance changes with these cell lines, the starting number of cells per well should be 5,000/well or greater and Biolog Redox Dye Mix MB should be employed (Table 1). Individual cell lines will vary and a graph of A_{590} versus cell number should be generated for each.

g. Cell Number Optimization

Cell proliferation assays require cells to grow over a period of time. Therefore, at the beginning of the culture period, select a titer of cells per well that produces a low, but detectable A_{590} value. This helps to ensure that the signal measured at the end of the assay will not exceed the linear range of the assay. This cell number should be empirically determined for each cell line by performing a cell titration, as described in section III.a.

Different cell types have different levels of metabolic activity. Factors that affect the metabolic activity of cells may affect the relationship between cell number and formazan production. Anchorage-dependent cells that undergo contact inhibition may show an alteration in metabolic activity per cell at high densities, resulting in a nonlinear relationship between cell number and formazan formation. Factors that affect the cytoplasmic volume or physiology of the cells may also affect metabolic activity.

V. References

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