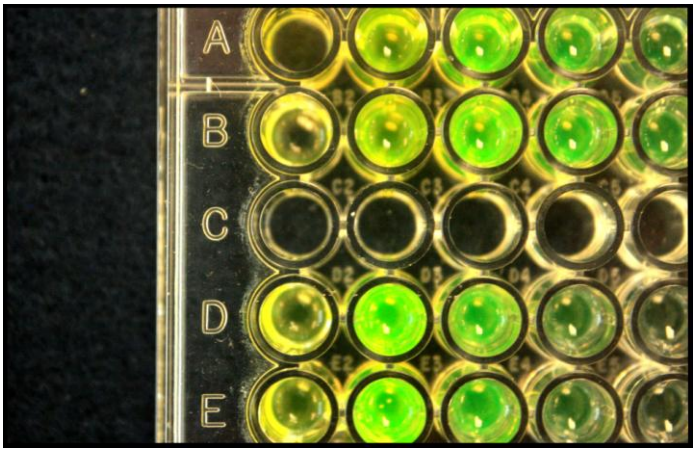
PRODUCT INFORMATION SHEET



MARKERGENE™ FLUORESCENT BACTERIAL DETECTION AND QUANTIFICATION KIT

(Product M1460)

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MarkerGene™ Fluorescent Bacterial Detection and Quantification Kit

(PRODUCT M1460)

NOTE: The following information is given as a viable methodology for use of the MarkerGene™ Fluorescent Bacterial Detection and Quantification Kit. The user may determine their own best methods for use dependent on the specific conditions present in their experiment.

I. OVERVIEW

Several isoforms of gram negative bacteria have specific enzyme activities that can be used to identify and quantify their presence at extremely low levels of cells. The *E.* coli *lac* operon which codes for the enzyme β-galactosidase, has been one of the most closely examined gene regulation and expression systems in molecular genetics. The operon exists in *E. Coli* to regulate transport and metabolism, and is regulated tightly by the concentrations of lactose and glucose in media or extracellular fluid. In native *E. coli* and in other coliform bacteria (see **Table 1**), the enzyme is only expressed at basal levels in the absence of lactose or IPTG (isopropyl 1-thio-galactoside) induction, and is difficult to detect due to this tightly regulated control.

In this kit, we use the highly sensitive substrate fluorescein di-β-D-galactopyranoside (FDG, **Figure 1**), a di-β-galactoside conjugate of the dye fluorescein to sensitively detect *E. Coli* and other coliforms through induced β-galactosidase activity.

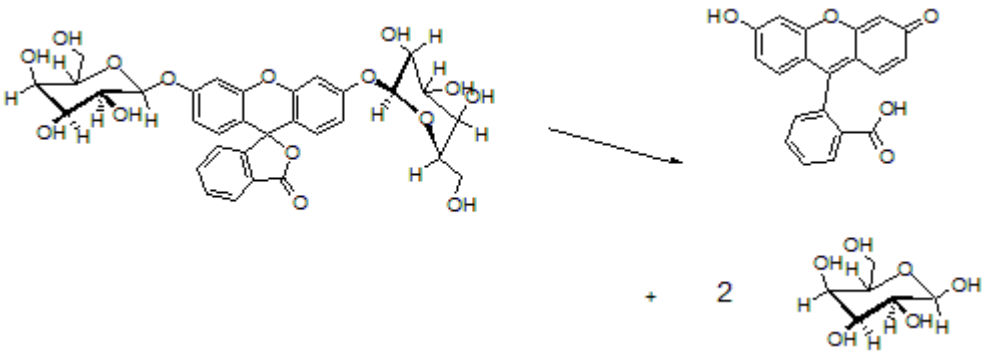


Figure. 1:
Fluorescein di-ß-D-Galactopyranoside (FDG) Breakdown to fluorescent Fluorescein.

Lactose acts as an inducer by binding the *lacI* repressor and releasing it from its binding site, effectively preventing *lacZ* inhibition and inducing production of β-galactosidase. The system naturally allows negative feedback by preventing production of the lactose metabolizing β-galactosidase enzyme in the absence of lactose as a sugar source.

Table 1. β-Gal Expressing Bacterial Strains

Strain	***Code***
Bijidobacterium bifidum	CUETM 89127
	CUETM 89/291
	CUETM 89/278
	CUETM 89/286
	ATCC 15696
Bijdobacterium breve	CUETM 89/220
	CUETM 89/160
	CUETM 90/103 T
Bijidobacterium longum or infantis	CUETM 89/ 157
	CUETM 89/ 177
	CUETM 89/216
	CUETM 89/276
Bifdobacterium longum	CUETM 89/11 T
Bijdobacterium infanris	CUETM 89/19 T
Bifdobacterium adolescentis	CUETM 89/14 T
Bifidobacterium angulatum	CUETM 89/12 T
	ATCC 27669
Bifidobacterium catenulatum	CUETM 89/96 T
	CUETM 90/ 136
	CUETM 89/158
Bifidobacterium dentium	CUETM 89/20 T
	ATCC 27679
	CUETM 89/141
	CUETM 89/219
Bijidobacterium pseudocatenulatum	CUETM 89/16 T
	CUETM 89/29
	CUETM 89/178
Ruminococcus lactaris	ATCC 29176
Ruminococcus gnavus	ATCC 29149
Bacteroides fragilis	ATCC 25285
	AM2
Lactobacillus sp.	LB4
	LB8
Lactobacillus plantarum	CIP 7139
Lactobacillus brevis	CIP 7135
Zactobacillus acidophilrrs	IPL 1627
Clostridium perfringens	ATCC 2636
Escherichia coli	CIP 54127

By using the fluorescent substrate FDG, detection levels for the enzyme can be 3-4 orders of magnitude more sensitive that either chromogenic substrates (like X-Gal) or UV absorption methods (such as o-nitrophenyl-β-D-galactoside, ONPG). Therefore, the detection and quantitation of bacteria, even without induction, can be attained down to 1 CFU.

II. MATERIALS

A.) 1 **FDG Substrate Buffer (#1460-001):** 5 mL of 1mg / mL FDG in 1% EtOH, 1% DMSO, in deionized H_2O. Sterile.

B.) 2 **Tubes for frozen Aliquots (#1460-002):** 5x 1.5 mL sterile tubes for preparation of aliquots for cold storage.

C.) 3 **LTB Growth Media (#1460-003)**: 17.8g of LTB broth powder for preparation of sample cultures. (17.8 g makes 0.5 L of LTB Media)

D.) 4 **Standard Solution A: (#1460-004):** 1.0 mL of 1mg / mL Fluorescein in 1% EtOH, 1% DMSO in deionized H_2O (Non-sterile).

E.) 5 **Wild Type *E. Coli* W3110 (#1460-005):** Agar slant of *Escherichia Coli* strain W3110 for purposes of generating a standard curve. The vial needs to either be immediately transferred to cold storage at or below -20°C or used to make agar slants or plate stocks as per standard microbiological technique.

F.) **Sterile Swabs:** for use in sampling surfaces or tissues. **(Bectin-Dickinson #220518 or equivalent, not provided).**

G.) **Rich LB media Agar plates (not provided).**

H.) **0.5% NaCl (Not Provided): See Appendix.**

Storage and Handling. Substrate solutions, buffers and aliquots should be stored at -20°C for storage. All materials should be handled with care. Unopened reagents should be stable for at least 6 months following purchase. High background readings for blank samples may indicate decomposition. Please consult our technical assistance desk for more information about use and storage conditions.

III. ASSAY CONDITIONS

A. DETECTION ASSAY CONDITIONS

It is recommended that measurements be made in triplicate, if possible. The assay monitors fluorescence at EX 495 nm and EM 515 using a microplate assay format. Other fluorescein filter sets with similar wavelengths can also be used. The user is asked to consult with the manufacturer (or instrument manual) for the particular instrument in use for appropriate setup conditions needed for these measurements.

For bacterial detection of very low CFU samples, cell-free blank wells for FDG background fluorescence controls should be measured. These background readings should be made in triplicate and run in parallel to the sample as described in the detection protocol (**IV**).

Appropriate volumes of Sterile LTB Broth (25 ml per test) will need to be prepared and autoclaved for bacterial cultures. See Appendix for other recipes. Please refer to manufacturer's instructions for autoclave use.

B. QUANTITATIVE ASSAY CONDITIONS

In addition to cell-free background fluorescence controls, for quantitative measurement of optically dense cultures, it is recommended to perform UV (light scatter) readings as detailed in the quantitative protocol.

NOTE: For optional verification of technique or to serve as a positive control, a sample of wild-type *E. Coli* (Strain W3110) [5] has been included (**#1460-005**). The stock can be used to inoculate an overnight seed culture which is then used to seed the assay culture as in Section **IV** step 2a, or Section **V** step 1a.

For optional conversion of relative fluorescence data into actual millimolar turnover of dye, a standard solution of Fluorescein (**#1460-004**) [4] (product in **Figure 1**) is included for generation of a standard curve for conversion.

IV. DETECTION ASSAY PROTOCOL

1.) Prepare a 25ml flask of sterile **LTB Growth Media** by sterile transfer of the pre-prepared LTB Media stock.

The sample intended for testing is used to seed a 25 ml culture of LTB media broth prepared in Step 1 using appropriate sterile technique. After inoculation, cultures are incubated at 37°C in a shaking water bath for 4-6 hours or until they are in exponential growth phase (OD_{600} ≥ 0.3).

NOTE: Cultures inoculated from already established stationary seed cultures will enter exponential growth within as short as an hour, while those seeded by bacterial swab, scrape, or single colony transfer from an agar plate will usually take longer to reach this growth phase (4-6 hours) depending on the number of CFU transferred. Refer to reference (8) for more information on bacterial growth curves.

2.) Inoculation Methods

- **a.** Seed culture: Sterile transfer 0.5 mL of a seed culture into 25mL LTB Media. (see Section V below for Quantitation).
- **b.** Bacterial swabs: Swab sample with sterile swab and drop into culture flask containing LTB media **(#1460-003)** 25 mL.
- **c.** Agar Plate: Use sterile transfer technique to scrape a single colony from an agar plate. If a loop is used, it is dipped into the culture, if a pick or pipette tip is used, it can be dropped directly into a culture flask containing LTB media **(#1460-003)** 25 mL.
- **d.** Water Sample: Add 890 mg LTB Powder 3 directly to 25 mL water sample.

3.) The FDG Substrate Buffer 1 provided is at the working concentration. If the entire volume is not going to be used immediately, this buffer should be divided into aliquots 2, stored at -20°C and removed from cold storage individually before use. Aliquots are moved out of cold storage and allowed to thaw and reach room temperature without heating before use, as heating can cause decomposition of the substrate.

4.) The culture is homogenized by vortex mixing or inversion, and pipetted in triplicate into wells of a 96-well microplate, 135µL per well.

5.) Background controls for FDG Background fluorescence and light scatter from turbid solutions are prepared in triplicate as follows: Pipette 15µL of FDG Buffer 1 into wells containing 135µL cell free growth media.

6.) Set up the plate-reader for a kinetic fluorescent assay using excitation at 490 nm, and emission read at 515 nm with measurement each hour for 6 to 19 hours (overnight).

7.) Incubate the plate covered and maintained at 37°C in the plate reader and take readings hourly (for up to 19 hours or overnight) or until the data is of sufficient sensitivity as required (Refer to **Table 2**). Alternately, the plate can be stored in a 37°C incubator for 19 hours and an endpoint value read as above, without kinetic measurements.

8.) Subtract the Cell Free FDG Background Control well readings (averaged) from sample well readings. Wells containing remaining fluorescence readings equal to or greater than that of the background controls represent samples that contain bacterial contamination.

Table 2.

Detection Sensitivity (CFU)				
Assay Incubation Time	**0 Hrs**	**6 Hrs**	**15 Hrs**	**19 Hrs**
E Coli (Strain W3110)	N/A	10^4	10^2	10^0
Induced Wild Type E Coli * (Strain W3110)	10^6	10^3	10^1	10^0
***lacI*⁻ Constitutive Mutant † (Strain CSH37)**	10^4	10^3	10^0	10^0

* These bacteria are grown in culture in the presence of 1mM IPTG to induce *lacZ* expression.
† *lacI*- Mutants do not produce functional *lacZ* repressors, and are in a constitutive *lacZ* expression state.

V. QUANTITATIVE ASSAY PROTOCOL

OVERVIEW: For quantitative assays, bacteria intended for testing are used to seed a 10mL culture in LTB Growth Media using appropriate sterile technique.

In order to quantitate CFU, a series of dilutions are prepared and incubated from this culture, and the resulting positive wells are used with the provided MPN table (Appendix B) to generate a Most Probable Number of bacteria in the original sample.

1.) Prepare a 10 mL volume of sterile **LTB Growth Media** containing the sample by sterile transfer of the sample to the pre-prepared LTB Media stock as below.

Inoculation Methods

- **a.** Water Sample: Add 356 mg LTB Powder directly to 10 mL of water sample.
- **b.** Bacterial swabs: Swab sample with sterile swab and drop into culture flask containing LTB media 10 mL.
- **c.** Agar Plate: Use sterile transfer technique to scrape a single colony from an agar plate. If a loop is used, it is dipped into the culture, if a pick or pipette tip is used, it can be dropped directly into a culture flask containing LTB media 10 mL.
- **d.** Seed culture: Sterile transfer 0.5 ml of a seed culture into 10 mL LTB Growth Media.

2.) Determine the dilution medium as follows:

- **a.** If the sample is expected to have a low CFU count; a water sample, swab sample, or colony from an agar plate (a, b or c above) the dilution media used is **LTB Growth Media**
- **b.** If the sample is inoculated from an exponentially growing or saturated seed culture, then 0.5% NaCl should be used as the dilution media. (See Appendix A)

3.) Serial dilutions of culture are made by pipetting 1 mL of the culture into 9 mL dilution media (determined in Step 2 above). This first dilution is then repeated until a total of 6 serial dilutions are prepared.

4.) The dilutions are mixed by vortexing or inversion, and pipetted **in triplicate** into wells of a 96-well microplate, 135μL per well.

5.) Background controls for FDG Background fluorescence and light scatter from turbid solutions are prepared in triplicate as follows: Pipette 15μL of FDG Buffer 1 into wells containing 135μL cell free dilution media.

6.) Incubate the plate covered and maintained at 37°C for 18 hours.

After 18 hour incubation:

7.) Set up the plate-reader for a single-measurement fluorescent assay with excitation at 490 nm, and emission at 515 nm (overnight).

8.) Subtract the Cell Free FDG Background Control well readings (averaged) from sample well readings. Wells containing fluorescence readings equal to or greater than 1.3 times that of the background wells after 18 hours of incubation are considered positive.

9.) After incubation, the number of tubes showing growth is recorded. To estimate the number of organisms per ml of the sample, locate the three wells which show dilution "to extinction" – i.e., those wells which precede the lowest dilution still showing fluorescence.

10.) Use the **3-well MPN Table** (Appendix B) to determine the most probable number of bacteria in the original sample as follows:

- **a.** Determine the first triplicate set of wells that has no growth *in any of the three wells*, and observe the two dilutions previous to that dilution. For example, if the first triplicate set to have no growth is 10^{-5}, then you will be considering dilutions 10^{-3}, 10^{-4} and 10^{-5}.
- **b.** Record the number of wells from each of the three wells that has exhibited growth. For example, if dilutions 10^{-3}, 10^{-4}, and 10^{-5} show growth in 3 wells, 2 wells and 0 wells respectively, the numbers recorded are [3,2,0].
- **c.** Apply this number set to the most probable number table (Appendix B) to produce a CFU/ well value. Following the example above, the CFU / well value for the 10^{-4} dilution would be 0.93.
- **d.** This value represents the number of viable bacteria per well at the dilution of the middle tube from the set of 3 (in this case 0.93 CFU / well at the 10^{-4} dilution).
- **e.** The produced value is then multiplied by 7.41 (1000uL/135uL) to give CFU / mL, and by the dilution factor to produce the number of CFU / mL in the original sample. (In this example 0.93×10^4 to give 9,300 CFU / mL)

NOTE for Steps 11-14: The sample concentration can be verified by plating the dilutions onto Agar plates with incubation and the number of colonies (CFU) counted for the lowest diluted samples:

11.) 100µL volumes of each serial dilution sample are sterile transferred to rich media Agar plates (not provided) and spread using a sterilized glass–rod spreader or other technique.

12.) Plates are incubated overnight and counts are taken from plates within 30-300 CFU / plate.

13.) The counted CFU / plate (CFU/100µL) is used to extrapolate a linear graph of CFU versus dilution factor from initial culture. This value is then used against the final fluorescence or OD readings of undiluted sample culture. The assay RFU readings from Step IV produce a direct relationship between fluorescence readings from your plate reader and CFU.

14.) A standard curve like that shown in **Figure 2** is used to determine the CFU vs. RFU for data generated in section IV above.

NOTE: For quantitative assays on optically dense cultures, background controls for FDG background fluorescence and for light scatter are prepared in triplicate as follows:

15.)

a. Cell Free FDG Background*: 15µL FDG Buffer 1 is pipetted into wells containing 135µL cell free growth media. Subtract this RFU reading as the background fluorescence adjustment.

* FDG can decompose in aqueous solution to the fluorescein fluorophore if not stored under appropriate conditions.

b. Light Scatter background: For optically dense cultures, include control wells containing the culture without substrate to account for any scattered light picked up by the plate reader. Substitute 15µL of deionized H_20 to these control wells in place of Substrate Buffer. This can also be done regardless of optical density as an assay control to show control RFU values produced by the plate reader in the absence of fluorescent substrate.

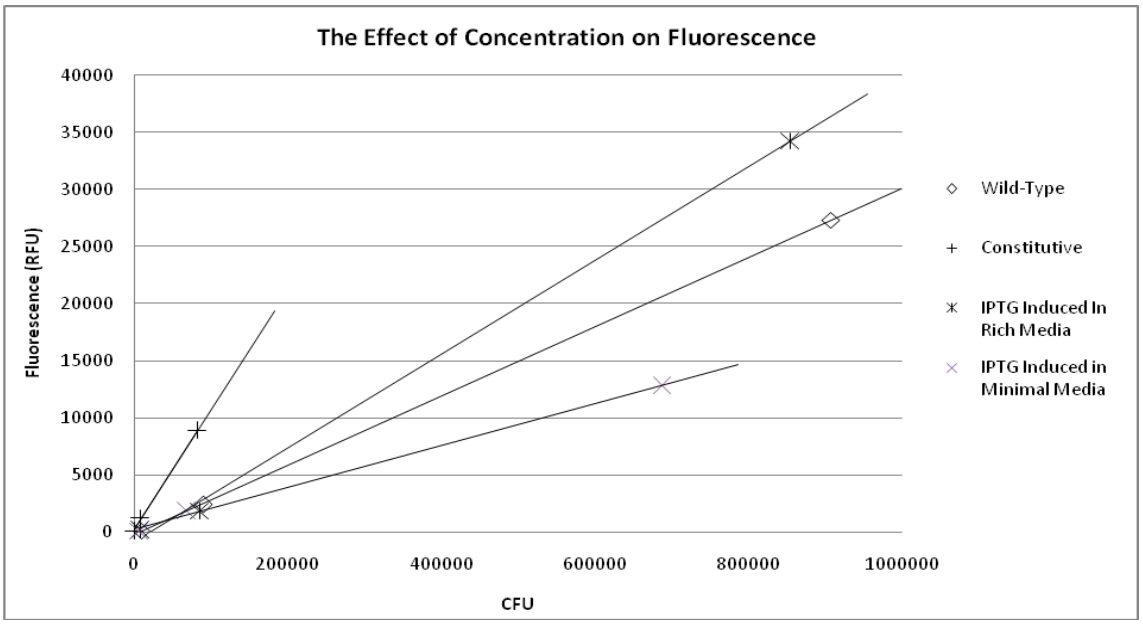
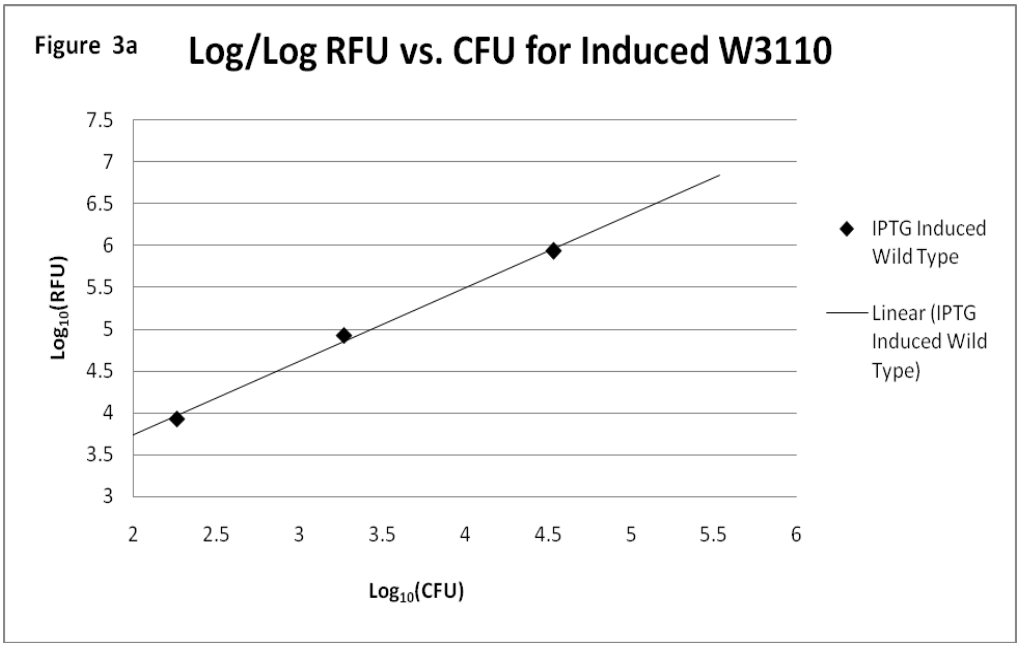


Figure 2. Fluorescence response produced from un-incubated dilutions of *E. coli* bacteria. This data does not reflect the actual sensitivity of the assay.



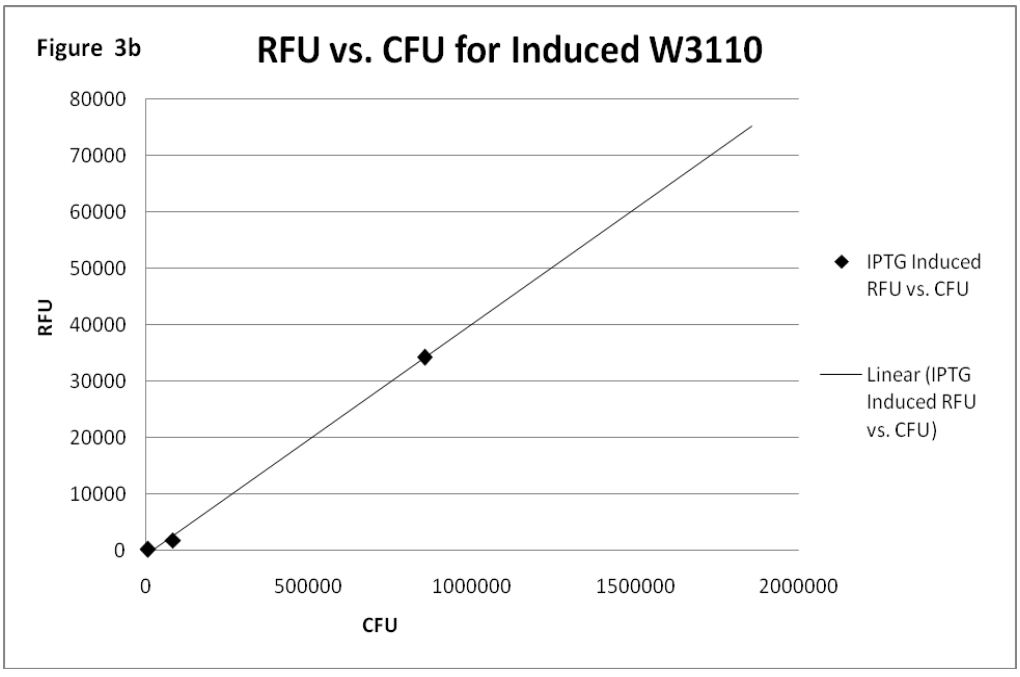


Figure 3a and 3b. Fluorescence response for low CFU bacterial samples. This data is given as a representative example of the assay. Your data will be dependent upon the specific microplate reader and conditions used.

REFERENCES:

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2.) University of Tasmania School of Biomedical Science, Microbiology Techniques Manual. Version 5.0, June 2002.

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5.) Nelis, H.; Van Poucke, S. “Enzymatic detection of coliforms and Escherichia coli within 4 hours." Water, Air, and Soil Pollution (2000), 123(1-4): 43-52.

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9.) Tao He, Marion G. Priebe, Roel J. Vonk, Gjalt W. Welling. (2005) “Identification of bacteria with β-galactosidase activity in faeces from lactase non-persistent subjects.” FEMS Microbiology Ecology 54: 463-469.

M1460 KIT CONTENTS			
DESCRIPTION	QUANTITY	PART NO.	STORAGE
1 FDG Substrate Buffer	1 X 5 mL	1460-001	F
2 Aliquot Tubes	5 X TUBES	1460-002	N/A
3 LTB Growth Media Powder	1 X VIAL	1460-003	R
4 Standard Solution A	1 mL AT 1MG / mL	1460-004	F
5 Wild Type *E. Coli*	1 SLANT	1460-005	C
DOCUMENTATION			
MSDS SHEETS	2	1460-006	
PRODUCT INFORMATION SHEET	1	1460-007	

Notes: F=store at or below -20^{o}C; R=store at room temperature; C=store cold (4^{o}C); L=light sensitive; D=store desiccated; FL=flammable; G=wear protective clothing/gloves/safety glasses when using; B=avoid breathing dust/fumes.

Appendix A:

0.5% NaCl Solution

Per Liter:

To 750 ml of sterile DI H_20, add 0.5g NaCl, dissolve and adjust volume to 1 L, and sterilize by autoclaving.

LTB Medium (Lauryl Tryptose Broth) Medium

Per Liter:

To 400 mL of DI water add 17.8g of LTB Media Powder [3]. Adjust this ratio to the desired volumes.

Shake or sonicate until solutes have dissolved, adjust to 500 mL final volume with DI water, and sterilize by autoclaving.

LB Agar Plate Recipe:

Per Liter:

To 950 ml of DI H_20, add:

Tryptone	10 g
Bacto Yeast Extract	5 g
NaCl	10 g
Agarose	15 g

Shake or sonicate until solutes have dissolved, Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 L with DI H20, and sterilize by autoclaving.

Appendix B: Most Probable Number (MPN) Statistical Table-triplicate wells.

First Set of Wells	Middle Set of Wells	Last Set of Wells	MPN of CFU / well for middle dilution set
0	0	0	‹0.03
0	0	1	0.03
0	0	2	0.06
0	0	3	0.09
0	1	0	0.03
0	1	1	0.061
0	1	2	0.092
0	1	3	0.12
0	2	0	0.062
0	2	1	0.093
0	2	2	0.12
0	2	3	0.16
0	3	0	0.094
0	3	1	0.13
0	3	2	0.16
0	3	3	0.19
1	0	0	0.036
1	0	1	0.072
1	0	2	0.11
1	0	3	0.15
1	1	0	0.073
1	1	1	0.11
1	1	2	0.15
1	1	3	0.19
1	2	0	0.11
1	2	1	0.15
1	2	2	0.2
1	2	3	0.24
1	3	0	0.16
1	3	1	0.2
1	3	2	0.24
1	3	3	0.29
2	0	0	0.091
2	0	1	0.14
2	0	2	0.2
2	0	3	0.26
2	1	0	0.15
2	1	1	0.2
2	1	2	0.27
2	1	3	0.34
2	2	0	0.21
2	2	1	0.28
2	2	2	0.35
2	2	3	0.42
2	3	0	0.29
2	3	1	0.36
2	3	2	0.44
2	3	3	0.53
3	0	0	0.23
3	0	1	0.39
3	0	2	0.64
3	0	3	0.95
3	1	0	0.43
3	1	1	0.75
3	1	2	1.2
3	1	3	1.6
3	2	0	0.93
3	2	1	1.5
3	2	2	2.1
3	2	3	2.9
3	3	0	2.4
3	3	1	4.6
3	3	2	11
3	3	3	›24

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