WCSB, Wageningen University	IBISBA-SOP-WU24
	Version 1.0

EPP - Standard Operating Procedure

(only for selected experiments intended to transfer results from one lab to the other)

Title: *Live/dead staining of bacteria*

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Experimenter 1	Rita Volkers		26/3/2019

Instruction

Live/dead staining of bacteria

1. Introduction / Purpose

This is a description of the protocol of the **LIVE/DEAD® BacLight™ Bacterial Viability Kits** by Molecular Probes. There are two kits available:
L7007LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy*
L7012LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy and quantitative assays*

Keywords: Live/dead – staining

2. Equipment and chemicals

2.1. Equipment

2.2. Chemicals

Either one of these kits:

- L7007LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy*
- L7012LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy and quantitative assays*

2.3. Other materials

- Culture to be analysed

3. Procedures

Experimental Protocols, General Considerations

The following protocols are provided as examples to guide researchers in the development of their own bacterial staining procedures. Researchers at Molecular Probes have used these procedures and found them to be simple and reliable for both gram-positive and gram-negative bacteria.

Culture Conditions and Preparation of Bacterial Suspensions

Note: Care must be taken to remove traces of growth medium before staining bacteria with these kit reagents. The nucleic acids and other media components can bind the SYTO 9 and propidium iodide dyes in unpredictable ways, resulting in unacceptable variations in staining. A single wash step is usually sufficient to remove significant traces of interfering media components from the bacterial suspension. Phosphate wash buffers are not recommended because they appear to decrease staining efficiency.

1.1 Grow 30 mL cultures of either *Escherichia coli* or *Staphylococcus aureus* to late log phase in nutrient broth (e.g., DIFCO catalog number 0003-01-6).

1.2 Concentrate 25 mL of the bacterial culture by centrifugation at 10,000 × g for 10–15 minutes.

1.3 Remove the supernatant and resuspend the pellet in 2 mL of 0.85% NaCl or appropriate buffer.

1.4 Add 1 mL of this suspension to each of two 30–40 mL centrifuge tubes containing either 20 mL of 0.85% NaCl or appropriate buffer (for live bacteria) or 20 mL of 70% isopropyl alcohol (for killed bacteria).

1.5 Incubate both samples at room temperature for 1 hour, mixing every 15 minutes.

1.6 Pellet both samples by centrifugation at 10,000 × g for 10–15 minutes.

1.7 Resuspend the pellets in 20 mL of 0.85% NaCl or appropriate buffer and centrifuge again as in step 1.6.

1.8 Resuspend both pellets in separate tubes with 10 mL of 0.85% NaCl or appropriate buffer each.

1.9 Determine the optical density at 670 nm (OD_{670}) of a 3 mL aliquot of the bacterial suspensions in glass or acrylic absorption cuvettes (1 cm pathlength).

1.10 For suggested concentrations of *E. coli* or *S. aureus* suspensions, please refer to the section appropriate for your instrumentation: fluorescence microscope, fluorometer, fluorescence microplate reader or flow cytometer.

Bacteria That Have Been Tested

The LIVE/DEAD *Bac*Light Bacterial Viability Kits have been tested at Molecular Probes on the following bacterial species: *Bacillus cereus*, *B. subtilis*, *Clostridium perfringens*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Mycobacterium phlei*, *Pseudomonas aeruginosa*, *P. syringae*, *Salmonella oranienburg*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus* and *Streptococcus pyogenes*. All of these bacterial types have shown a good correlation between the results obtained with the LIVE/DEAD *BacLight* Bacterial Viability Kits and those obtained with standard plate counts. These tests were performed on logarithmically growing cultures of organisms. In addition, we have received favorable reports from researchers who have used these kits with: *Agrobacterium tumefaciens*, *Edwardsiella ictaluri*, *Eurioplasma eurilytica*, *Lactobacillus* sp., *Mycoplasma hominus*, *Propionibacterium* sp., *Proteus mirabilis* and *Zymomonas* sp.

Optimization of Staining

The two dye components provided with the LIVE/DEAD *Bac*Light Bacterial Viability Kits have been balanced so that a 1:1 mixture provides good live/dead discrimination in most applications. Occasionally, however, the proportions of the two dyes must be adjusted for optimal discrimination. For example, if green fluorescence is too prominent in the preparation, we suggest that you try either lowering the concentration of SYTO 9 stain (by using less of Component A) or by raising the concentration of propidium iodide (by using more of Component B).

To thoroughly optimize the staining, we recommend experimenting with a range of concentrations of SYTO 9 dye, each in combination with a range of propidium iodide concentrations. In the case of Kits L7007 and L7012, you may wish to try staining 1.0 mL of the bacterial suspension with 3 µL of dye pre-mixed at different Component A:Component B ratios. In the case of kit L13152, separate dye solutions can be made by dissolving the contents of one Component A pipet in 2.5 mL filter-sterilized dH_2O and the contents of one Component B pipet in 2.5 mL filter-sterilized dH_2O. These separate solutions can be blended at different ratios, and then the mixtures applied 1:1 with the bacterial suspension.

Fluorescence Microscopy Protocols

Selection of Optical Filters

The fluorescence from both live and dead bacteria may be viewed simultaneously with any standard fluorescein longpass filter set. Alternatively, the live (green fluorescent) and dead (red fluorescent) cells may be viewed separately with fluorescein and Texas Red bandpass filter sets. A summary of the fluorescence microscope filter sets recommended for use with the LIVE/DEAD *Bac*Light Bacterial Viability Kits shown in Table 1.

Staining Bacteria in Suspension with either Kit L7007 or L7012

2.1 Combine equal volumes of Component A and Component B in a microfuge tube, mix thoroughly.

2.2 Add 3 µL of the dye mixture for each mL of the bacterial suspension. When used at the recommended dilutions, the reagent mixture will contribute 0.3% DMSO to the staining solution. Higher DMSO concentrations may adversely affect staining.

2.3 Mix thoroughly and incubate at room temperature in the dark for 15 minutes.

2.4 Trap 5 µL of the stained bacterial suspension between a slide and an 18 mm square coverslip.

2.5 Observe in a fluorescence microscope equipped with any of the filter sets listed in Table 1.

Staining Bacteria in Suspension with Kit L13152

3.1 Prepare a 2X stock solution of the LIVE/DEAD *Bac*Light staining reagent mixture by dissolving the contents of one Component A pipet (containing yellow-orange solids) and one Component B pipet (containing red solids) in a common 5 mL–volume of filter-sterilized dH_2O.

Table 1. *Characteristics of common filters suitable for use with the LIVE/DEAD BacLight Bacterial Viability Kits.*

Omega Filters*	**Chroma Filters***	**Notes**
XF25, XF26, XF115	11001, 41012, 71010	Longpass and dual emission filters useful for simultaneous viewing of SYTO 9 and propidium iodide stains
XF22, XF23	31001, 41001	Bandpass filters for viewing SYTO 9 alone
XF32, XF43 XF102, XF108	31002, 31004 41002, 41004	Bandpass filters for viewing propidium iodide alone

***** Catalog numbers for recommended bandpass filter sets for fluorescence microscopy. Omega filters® are supplied by Omega Optical Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).

3.2 Combine a sample of the 2X stock solution with an equal volume of the bacterial suspension. The final concentration of each dye will be 6 µM SYTO 9 stain and 30 µM propidium iodide.

3.3 Mix thoroughly and incubate at room temperature in the dark for 15 minutes.

3.4 Trap 5 µL of the stained bacterial suspension between a slide and an 18 mm square coverslip.

3.5 Observe in a fluorescence microscope equipped with any of the filter sets listed in Table 1.

Fluorescence Spectroscopy Protocols

Staining Bacteria with either Kit L7007 or L7012

4.1 Adjust the *E. coli* suspensions (live and killed) to 1×10^8 bacteria/mL (~0.03 OD_{670}) or the *S. aureus* suspensions (live and killed) to 1×10^7 bacteria/mL (~0.15 OD_{670}). *S. aureus* suspensions typically should be 10-fold less concentrated than *E. coli* for fluorescence spectroscopy.

4.2 Mix five different proportions of the bacterial suspensions in 1 cm acrylic, glass or quartz fluorescence cuvettes (Table 2). The total volume of each of the five samples will be 3 mL.

4.3 Prepare a combined reagent mixture in a microfuge tube by adding 30 µL of Component A to 30 µL of Component B.

4.4 Add 9 µL of the combined reagent mixture to each of the five samples (5 samples × 9 µL = 45 µL total) and mix thoroughly by pipetting up and down several times.

4.5 Incubate at room temperature in the dark for 15 minutes.

Staining Bacteria with Kit L13152

5.1 Adjust the *E. coli* suspensions (live and killed) to 2×10^8 bacteria/mL (~0.06 OD_{670}) or the *S. aureus* suspensions (live and killed) to 2×10^7 bacteria/mL (~0.30 OD_{670}). *S. aureus* suspensions typically should be 10-fold less concentrated than *E. coli* for fluorescence spectroscopy.

Component A pipet (containing yellow-orange solids) and one Component B pipet (containing red solids) in a common 5 mL–volume of filter-sterilized dH_2O.

5.2 Mix five different proportions of the bacterial suspensions in 1 cm acrylic, glass or quartz fluorescence cuvettes (Table 2). Note that when using kit L13152, only one-half of the cell suspension volume (1.5 mL) listed in Table 2 will be used.

5.3 Prepare a 2X working solution of the LIVE/DEAD *Bac*Light staining reagent mixture by dissolving the contents of one

between 620–650 (em2; red) for each bacterial suspension.

$$\text{Ratio}_{G/R} = \frac{F_{cell,em1}}{F_{cell,em2}}$$

5.4 Mix 1.5 mL of the 2X staining reagent mixture with an equal volume (1.5 mL) of each bacterial suspension. Note that, as described above, two applicator sets will be needed (5 samples × 1.5 mL = 7.5 mL total); however, it may be possible to use smaller volumes.

5.5 Incubate at room temperature in the dark for 15 minutes.

Fluorescence Spectroscopy and Data Analysis

6.1 Measure the fluorescence emission spectrum (excitation 470 nm, emission 490–700 nm) of each cell suspension (F_{cell}) in a fluorescence spectrophotometer (Figure 1a).

6.2 Calculate the ratio of the integrated intensity of the portion of each spectrum between 510–540 nm (em1; green) to that

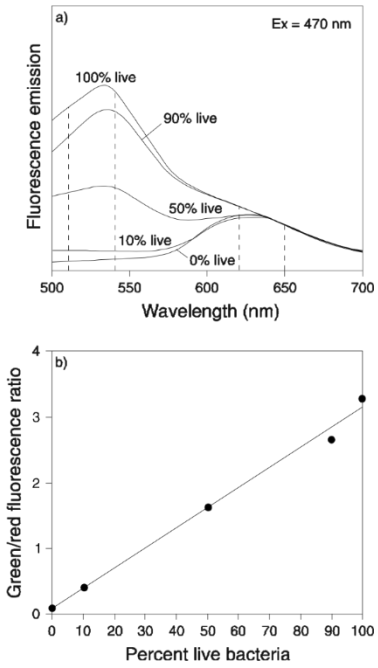

Figure 1. *Analysis of relative viability of E. coli suspensions by fluorescence spectroscopy. a) Emission spectra of suspensions of various proportions of live and isopropyl alcohol–killed E. coli were obtained from samples prepared and stained as outlined in the text. Integrated fluorescence emission intensities were determined from the spectral regions indicated by dashed vertical lines. b) Integrated intensities of the green (510–540 nm) and red (620–650 nm) emission were acquired, and the green/red fluorescence ratios ($Ratio_{G/R}$) were calculated for each proportion of live/dead E. coli. The line is a least-squares fit of the relationship between % live bacteria (x) and $Ratio_{G/R}$ (y).*

Table 2.
Volumes of live- and dead-cell suspensions to mix to achieve various proportions of live:dead cells for fluorescence spectroscopy.

Ratio of Live:Dead Cells	mL Live-Cell Suspension	mL Dead-Cell Suspension
0:100	0	3.0
10:90	0.3	2.7
50:50	1.5	1.5
90:10	2.7	0.3
100:0	3.0	0

6.3 Plot the ratio of integrated green fluorescence to integrated red fluorescence ($R_{G/R}$) versus percentage of live cells in the *E. coli* suspension (Figure 1b).

Fluorescence Microplate Readers

Conditions required for measurement of fluorescence in microplate readers are very similar to those required for fluorescence spectroscopy of bacterial cell suspensions. As in fluorescence spectroscopy experimental protocols, reagent concentrations are the same as those recommended for fluorescence microscopy, and the ratio of green to red fluorescence emission is proportional to the relative numbers of live bacteria.

Staining Bacterial Suspensions with either Kit L7007 or L7012

7.1 Adjust the *E. coli* suspensions (live and killed) to 2×10^8 bacteria/mL (~0.06 OD_{670}) or the *S. aureus* suspensions (live and killed) to 2×10^7 bacteria/mL (~0.30 OD_{670}). *S. aureus* suspensions typically should be 10-fold less concentrated than *E. coli* when using a fluorescence microplate reader. recommend that you prepare samples in triplicate. The outside wells (rows A and H and columns 1 and 12) are usually kept empty to avoid spurious readings.

Table 3. *Volumes of live- and dead-cell suspensions to mix to achieve various proportions of live:dead cells for fluorescence microplate readers.*

Ratio of Live:Dead Cells	mL Live-Cell Suspension	mL Dead-Cell Suspension
0:100	0	2.0
10:90	0.2	1.8
50:50	1.0	1.0
90:10	1.8	0.2
100:0	2.0	0

7.2 Mix five different proportions of *E. coli* or *S. aureus* (Table 3) in 16 × 125 mm borosilicate glass culture tubes. The total volume of each of the five samples will be 2 mL.

7.3 Mix 6 µL of Component A with 6 µL of Component B in a microfuge tube.

7.4 Prepare a 2X stain solution by adding the entire 12 µL of the above mixture to 2.0 mL of filter-sterilized dH_2O in a 16 × 125 mm borosilicate glass culture tube and mix well.

7.5 Pipet 100 µL of each of the bacterial cell suspension mixtures into separate wells of a 96-well flat-bottom microplate. We

7.6 Using a new tip for each well, pipet 100 µL of the 2X staining solution (from step 7.4) to each well and mix thoroughly by pipetting up and down several times.

7.7 Incubate at room temperature in the dark for 15 minutes.

Staining Bacterial Suspensions with Kit L13152

8.1 Adjust the *E. coli* suspensions (live and killed) to 4×10^8 bacteria/mL (~0.12 OD_{670}) or the *S. aureus* suspensions (live and killed) to 4×10^7 bacteria/mL (~0.60 OD_{670}). *S. aureus* suspensions typically should be 10-fold less concentrated than *E. coli* when using a fluorescence microplate reader.

8.2 Mix five different proportions of *E. coli* or *S. aureus* (Table 3) in 16 × 125 mm borosilicate glass culture tubes.

8.3 Prepare a 2X working solution of the LIVE/DEAD *Bac*Light staining reagent mixture by dissolving the contents of one Component A pipet (containing yellow-orange solids) and one Component B pipet (containing red solids) in a common 5 mL–volume of filter-sterilized dH_2O.

8.4 Pipet 100 µL of each of the bacterial cell suspension mixtures into separate wells of a 96-well flat-bottom microplate. We recommend that you prepare samples in triplicate. The outside wells (rows A and H and columns 1 and 12) are usually kept empty to avoid spurious readings.

8.5 Using a new tip for each well, pipet 100 µL of the 2X working stain solution (from step 8.3) to each well and mix thoroughly by pipetting up and down several times.

8.6 Incubate the sample at room temperature in the dark for 15 minutes.

Fluorescence Measurement and Data Analysis

9.1 With the excitation wavelength centered at about 485 nm, measure the fluorescence intensity at a wavelength centered at about 530 nm (emission 1; green) for each well of the entire plate.

9.2 With the excitation wavelength still centered at about 485 nm, measure the fluorescence intensity at a wavelength centered about 630 nm (emission 2; red) for each well of the entire plate.

9.3 Analyze the data by dividing the fluorescence intensity of the stained bacterial suspensions (F_{cell}) at emission 1 by the fluorescence intensity at emission 2.

$$\text{Fcell,em1 Ratio}_{G/R} = \frac{\text{Fcell,em1}}{\text{Fcell,em2}}$$

9.4 Plot the $Ratio_{G/R}$ versus percentage of live cells in the *E. coli* suspension (Figure 2).

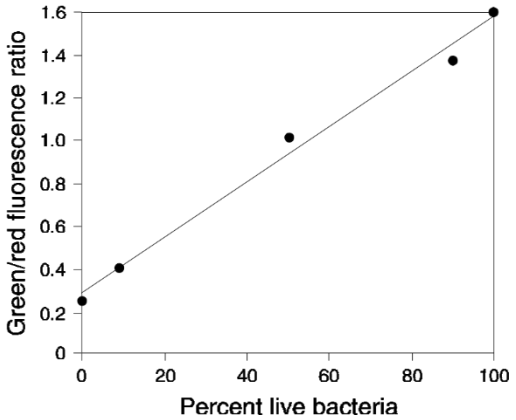

Figure 2. *Analysis of relative viability of E. coli suspensions in a fluorescence microplate reader. Samples of E. coli were prepared and stained as outlined in the text. The integrated intensities of the green (530 ± 12.5 nm) and red (620 ± 20 nm) emission of suspensions excited at 485 ± 10 nm were acquired, and the green/red fluorescence ratios ($Ratio_{G/R}$) were calculated for each proportion of live/dead E. coli. Each point represents the mean of ten measurements. The line is a least-squares fit of the relationship between % live bacteria (x) and $Ratio_{G/R}$ (y).*

Flow Cytometry

Instrument capabilities may vary considerably but the techniques and parameters established here should aid considerably in setting up similar analyses in the majority of flow cytometers now in use, both in research and clinical environments.

Table 4. *Volumes of live- and dead-cell suspensions to mix to achieve various proportions of live:dead cells for flow cytometry.*

Ratio of Live:Dead Cells	mL Live-Cell Suspension	mL Dead-Cell Suspension
0:100	0	2.0
10:90	0.2	1.8
20:80	0.4	1.6
30:70	0.6	1.4
40:60	0.8	1.2
50:50	1.0	1.0
60:40	1.2	0.8
70:30	1.4	0.6
80:20	1.6	0.4
90:10	1.8	0.2
100:0	2.0	0

Staining Bacterial Suspensions with either Kit L7007 or L7012

10.0 Dilute Component A and Component B 1:20 in MQ

10.1 Adjust the *E. coli* suspensions (live and killed) to 1×10^8 bacteria/mL (~0.03 OD_{670}), then dilute them 1:100 in filtersterilized dH_2O to reach a final density of 1×10^6 bacteria/mL.

10.2 Mix 11 different proportions of *E. coli* in 16 × 125 mm borosilicate glass tubes according to Table 4. The volume of each of the 11 samples will be 2 mL.

10.3 Mix 35 µL of Component A with 35 µL of Component B in a microfuge tube. If Kit L7012 is used, it may be desirable to prepare additional bacterial samples for staining with Component A alone and with Component B alone.

10.4 Add 6 µL of the combined reagent mixture to each of the 11 samples (11 samples x 6 µL = 66 µL total) and mix thoroughly by pipetting up and down several times.

10.5 Incubate at room temperature in the dark for 15 minutes.

Staining Bacterial Suspensions with Kit L13152

11.1 Adjust the *E. coli* suspensions (live and killed) to 1×10^8 bacteria/mL (~0.03 OD_{670}), then dilute them 1:50 in filtersterilized dH_20 to reach a final density of 2×10^6 bacteria/mL.

11.2 Mix 11 different proportions of *E. coli* in 16 × 125 mm borosilicate glass tubes according to Table 4. Note that when using kit L13152, only one-half of the cell suspension volume (1.0 mL) listed in Table 3 will be used.

11.3 Prepare a 2X working solution of the LIVE/DEAD *Bac*Light staining reagent mixture by dissolving the contents of one Component A pipet (containing yellow-orange solids) and one Component B pipet (containing red solids) in a common 5 mL–volume of filter-sterilized dH_2O. It may be desirable to prepare additional bacterial samples for staining with Component A alone (dissolved in 5 mL of filter-sterilized dH_2O) and with Component B alone (dissolved in 5 mL of filter-sterilized dH_2O).

11.4 Mix 1 mL of the 2X working solution of the LIVE/DEAD *Bac*Light staining reagent mixture with an equal volume (1 mL) of the bacterial suspension. Note that, as described above, three applicator sets will be needed (11 samples × 1 mL = 11 mL total); however it may be possible to use smaller volumes.

11.5 Incubate the sample at room temperature in the dark for 15 minutes.

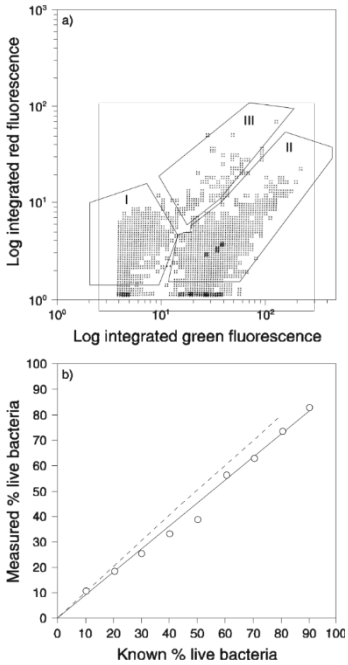

Figure 3. *Analysis of relative viability of E. coli suspensions by flow cytometry. Samples of E. coli were prepared, stained and analyzed as outlined in the text. a) A two-parameter comparison of the green and red components of fluorescence emission of individual bacteria from a population containing 70% "killed" organisms indicates two major regions (I & II) and one minor region (III). The majority of the bacteria are represented by region I (dead cells) and region II (live cells), which have similar red fluorescence intensity and different proportions of green fluorescence. E. coli organisms appearing in region III generally represent less than 5% of the population and are as yet uncharacterized in terms of viability. b) Known viability is defined as the proportion of "live" to "killed" bacteria. Measured viability is defined by the following equation: Measured % live bacteria = (# of bacteria in region II/# of bacteria in regions I+II) × 100. A least-squares fit extrapolated to the 100% "live" point suggested a 13%*

Instrument Parameters

The data shown in this example were acquired with a Coulter EPICS V™ flow cytometer equipped with an argon-ion laser at 488 nm and 400 mW output. Data acquisition and analysis were controlled using Cytomation CICERO software and a hardware interface. The emission light path contained a 515 nm blocking filter, 590 nm dichroic filter before the Green PMT and a 610 nm absorbance filter before the Red PMT. The density of the *E. coli* bacterial suspension was 1×10^6 cells/mL and the sampling rate was ~300 particles/sec. The sheath fluid was distilled water and the flow tip was a 76 µm air tip.

Fluorescence Measurements and Data Analysis

Because both live and dead cells exhibit green fluorescence, the signal discriminator was set at 15% of the log-integrated green fluorescence (LIGFL) to eliminate debris. Populations of bacteria were discriminated as three regions of the log-integrated red fluorescence (LIRFL) versus LIGFL plot (Figure 3a), and the numbers of bacteria found within these regions were used to estimate the percentage of viable organisms in the population (Figure 3b).

L7007	LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy* *1000 assays*	1 kit
L7012	LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy and quantitative assays* *1000 assays*.....	1 kit
L13152	LIVE/DEAD® BacLight™ Bacterial Viability Kit *10 applicator sets*	1 kit

4. Remarks / troubleshooting

This is the quick and short protocol:

- Dilute Component A and Component B 1:20 in MQ. Store in the dark @ -20˚C
- Spin 500µl culture down 5min @ max rpm, discard supernatant
- Wash with 20µl 0.9% Saline Solution
- Spin down 5min @ max rpm, discard supernatant
- Take up pellet in 20µl 0.9% Saline Solution
- Add 4µl PI and 1µl Syto9, Incubate in the dark for 10 min
- Analyse results with Flow Cytometry and/or fluorescence microscope

The long protocol can be found in section 3. (Procedures). It was copied from this website:

- https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp07007.pdf

5. Biosafety

No biosafety issues are associated with this protocol.

6. Acknowledgements

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