Shigella SBA against *Shigella flexneri* 2a, *Shigella flexneri* 3a and *Shigella sonnei*

(Version V.03, March 2018)

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www.vaccine.uab.edu/SBAProtocol.pdf

We thank help from Dr. R. Kaminski at WRAIR, Dr. H. Weerts at WRAIR, and Dr. H. Wenzel in PATH

Section 1. Equipment

- Biological safety cabinet
- Incubator, 37°C room air
- Incubator, 26°C
- Incubator, 29°C
- Microcentrifuge with rotor for 1.5-ml tubes, Heraeus Biofuge Fresco by Kendro Laboratory Products
- Mini-orbital shaker, Bellco Biotechnology 7644-20115
- Microtiter plate vortex mixer, Fisher 02206100
- Water bath, 56°C
- Multichannel pipettes, various sizes (including 0.1-10 microliter)

Section 2. Target Bacteria, Materials, and Reagents

- Target Bacterial strains

Shigella flexneri 2a	**2457T** from WRAIR
Shigella flexneri 3a	**J17B** from WRAIR
Shigella sonnei	**Moseley** from WRAIR

- Assay Plate: 96 well round bottom tissue culture plate with lid, Costar 3799
- Square Petri Dish (12cm X 12cm) manufactured by Greiner (688102), distributed by Sigma Z617679-240EA
- HBSS with $Ca^{++}$and Mg^{++}, 10X, Invitrogen 14065-56 (see Note 1)
- Gelatin, Sigma G9391, make 1% solution (See Prepared Reagents below)
- Baby rabbit complement (BRC), Pel-Freez®

- TTC (2,3,5,-triphenyltetrazolium chloride), Sigma T8877, make 100 mg/ml (See Prepared Reagents below)
- NaN_3 (Sodium azide), Sigma S2002, make 10% solution (See Prepared Reagents below and Note 5)
- Sterile reagent reservoir, Costar 4870
- Pipets, serological, sterile, various sizes
- Microcentrifuge tubes, 1.5 ml size
- Centrifuge tubes, 50 ml
- Pipet tips, various sizes
- Pipettes, various sizes
- Assay Buffer (See Prepared Reagents below)
- LBA plate (See Prepared Reagents below)
- Overlay Agar (See Prepared Reagents below)
- Heat-inactivated BRC (See Prepared Reagents below)
- Target Bacteria Working Stock (See Preparation of frozen Target Bacteria Stock)

Section 3. Prepared Reagents

1% Gelatin
Add 1 g of gelatin (Sigma G9391) to 100 ml of water. Autoclave and store at RT.

TTC
Prepare a 100 mg/ml (1000X) stock solution in water by adding 5 g of TTC (Sigma T8877) to 40 ml water. After dissolution, adjust volume to 50 ml with additional water and sterile filter using 0.2 micrometer filter. Store at 4^{o}C. Solution should have slight yellow color. If solution develops red color, discard and prepare new one. TTC colorizes the bacterial colonies and makes them much easier to count.

10% sodium azide (See Note 5)
Add 5 g NaN_3 (Sigma S2002) to 40 ml of water. After complete dissolution, add water to 50 ml.

Heat-inactivated BRC
Incubate BRC in 56^{o}C water bath for 30 minutes. Prepare 125 microliter aliquots and store at -80 ^{o}C.

Assay Buffer
Add 5 ml of 10X HBSS with Ca^{2+}/Mg^{2+} (Invitrogen 14065-56) and 5 ml of 1% gelatin to 40 ml of water.

LBA plate (LB agar plate)
Mix 35 g of LB agar (Sigma L2897) and 1L of water, and autoclave. Add 25 ml to each Petri dish (Sigma Z617679-240EA). Incubate plates at RT for 10-20 minutes to allow agar to solidify. Place plates back into plastic bags and store at 4°C for up to 1 month.

Overlay Agar

Add 6 g of agar (BD 214010) to 800 ml of water and autoclave. Incubate in 56°C water bath until needed. Before use, add 800 µl of 100 mg/ml TTC (1000-fold dilution of stock solution) and 8 ml of 10% NaN3 (100-fold dilution of stock solution) and mix well.

Section 4. Bactericidal Assay Procedure

The procedure described below is for one assay plate. See Note 2 for assays involving multiple plates.

A. If not already done, heat-inactivate test samples (see Note 3).
B. Prepare Assay Buffer (you will need 50 ml).
C. Prepare Overlay Agar (you will need 50 ml).
D. Dry LBA plates (you will need 2 plates) by removing lids and placing plates in biosafety cabinet for 60 minutes.
E. Get an Assay Plate. Add 20 µl of Assay Buffer to columns 1 through 12 of rows A through G. See Figure 1.
F. Add 20 µl of Assay Buffer to columns 1 and 2 of row H as well.
G. Load 30 µl of each test sample, in duplicate, to row H of the Assay Plate. Columns 3 and 4 will receive test sample 1, columns 5 and 6 will receive test sample 2, etc.

Figure 1. Assay Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control A	Control B	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8
B	Control A	Control B	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7
C	Control A	Control B	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
D	Control A	Control B	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5
E	Control A	Control B	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4
F	Control A	Control B	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3
G	Control A	Control B	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2
H	Control A	Control B	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1
			Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	

H. Prepare 3-fold serial dilutions of test samples (columns 3 through 12) by transferring 10 µl of sample from row H to row G (containing 20 µl of Assay Buffer), mixing well, transferring 10 µl to row F, etc. Continue through row A. After mixing the wells in row A, remove and discard 10 µl such that the final volume in all wells is 20 µl.
I. Remove a vial of frozen Target Bacteria Stock, thaw at room temperature.
J. Dilute the bacteria according to the pre-determined optimal dilution factor (see Section 5. Preparation of frozen Target Bacteria Stock). Add 10 µl of diluted bacteria to each wells of the assay plate.
K. Prepare a 20% **heat-inactivated** BRC solution by mixing 100 µl of heat-inactivated BRC with 400 µl of Assay Buffer. Add 50 µl of this mixture to all wells in column 1 (Control A wells).
L. Prepare a 20% **native** BRC solution by mixing 1 ml of native BRC with 4 ml of Assay Buffer. Add 50 µl of this mixture to all wells in columns 2 through 12 (Control B and test sample wells). (**NOTE** that the final percent of BRC in the reaction mixture is 12.5% of complement.)
M. Briefly mix Assay Plate by shaking at 700 rpm for 10-15 seconds.
N. Put Assay Plate in 37°C room air incubator for 2 hours (without shaking).
O. Incubate Assay Plate on ice for 10-20 minutes to stop the reaction.

P. Using a 12-channel pipette (0.1 to 10 µl) to mix the wells in row H, and transfer 10 µl and spot plate onto the bottom of an LBA plate. Immediately tilt the plate and allow the spots to run for ~1.5-2 cm. Repeat this procedure for row G, F, and E, spotting them next to the previous row on the LBA plate. Row E, F, G, and H are spotted onto one LBA plate, and row A, B, C, and D are spotted onto a second LBA plate. See Figure 2.

Q. Incubate the LBA plates in room air until the solution is adsorbed into the agar plates (10-15 minutes).

R. Place the LBA plates in an incubator (*S. flexneri* 2a and 3a at 29 ^{o}C, *S. sonnei* at 26 ^{o}C) in room air overnight (16 hours). This temperature yields colony sizes suitable for accurate counting by a colony counter.

S. After incubation, add 25 ml of Overlay Agar (at ~45°C) containing 100 µg/ml TTC and 0.1% NaN_3 to each LBA plate. (Please note 0.1% NaN_3 is on the high side but we found it to be preferable.)

T. Incubate the LBA plates at 37°C for 2 hours to allow the surviving bacteria to develop red color, see Figure 2 below.

U. Enumerate the surviving colonies using NICE (see Note 4).

V. Calculate the bactericidal titer, killing index (KI), using the average CFU/spot counts of the two duplicate wells. Bactericidal KI is defined as the reciprocal dilution of sample that gives 50% killing of controls. This 50% value is calculated by taking the average CFU/spot of Control B wells and dividing by two. The formula for calculating the 50% killing index (KI) is shown below:

$$\mathrm{KI} = 10^{\left[\log \mathrm{X1} + \frac{(\mathrm{Y50}-\mathrm{Y1})\ \mathrm{x}\ (\log \mathrm{X2}-\log \mathrm{X1})}{(\mathrm{Y2}-\mathrm{Y1})}\right]}$$

Section 5. Preparation of frozen Target Bacteria Stock

Preparation of Target Bacteria Stock

Aliquots of Target Bacteria Stocks can be produced and frozen. An aliquot of assay bacteria stock is thawed and used only once. The procedure below is for preparing 48 aliquots of assay bacteria stock. If more aliquots are desired, scale up accordingly.

A. Retrieve the bacteria master stock vial from the freezer, quickly remove a fleck of ice from the vial, and streak it onto a blood agar plate. Place the cover on the plate. Immediately return the master stock vial to the freezer.

B. Incubate the plate upside down overnight in 37°C/5% CO_2 incubator.

C. Transfer ~10 isolated smooth colonies to a 50 ml tube containing 30 ml of LB broth.

D. Incubate for 3-5 hours at 37°C with gently shaking until the culture broth has an OD_{600} of ~0.6-0.7.

E. Harvest the top 12.5 ml of the culture and transfer to a fresh 50 ml tube. Centrifuge the culture at ~6000 rpm for 2 minutes with Sorvall RC 6 Plus centrifuge. Discard the supernatant, re-suspend the pellet in 25 ml of 15% sterile glycerol-LB.

F. Mix well and dispense 0.5 ml aliquots into sterile 1.5 ml micro centrifuge tubes (~ 48 tubes).

G. Confirm the bacterial identity using the agglutination test before using it.

Determination of optimal dilution factor for Target Bacteria Stock

Each batch of Target Bacteria Stock must be titrated in assay conditions to determine the dilution necessary to yield ~120 CFU/spot on LB plates.

A. Prepare Assay Buffer (50 ml) and Overlay Agar (you will need 25 ml).
B. Dry LBA plates (you will need 1 plate) by removing lids and placing plates in biosafety cabinet for 60 minutes.
C. Prepare serial dilutions of Target Bacteria Stock: this can be conveniently prepared in a fresh microtiter plate (dilution plate) as below.
    i. Rapidly thaw a vial of frozen bacteria stock.
    ii. Dilute the bacteria 10-fold by mixing 15 µl of bacteria with 135 µl of Assay Buffer in columns 1 of row A.
    iii. Add 120 µl of Assay Buffer to columns 1, rows B through H.
    iv. Prepare 5-fold serial dilutions by mixing 30 µl of diluted bacteria from row A with 120 µl of Assay Buffer in row B.
    v. Continue 5-fold serial dilutions for a total of 8 dilutions: 10-fold to7.8 x 10^5-fold.
D. Add 20 µl of Assay Buffer to all rows of columns 1 and 2 in an assay plate.
E. Transfer 10 µl of the diluted bacteria from column 1 of the dilution plate to column 1 and 2 of the assay plate.
F. Continue with the assay as described above for Control A and Control B.
G. After the assay is complete and plates have been incubated on ice, use a multichannel pipette with 8 pipet tips to spot 10 µl from the wells in column 1 onto an LBA plate. Also, spot the wells from column 2 onto the LBA plate.
H. Continue with the assay as described above.
I. After color development, determine the bacteria dilution that yields ~120 CFU/spot in Control B.

Section 6. Assay Notes

Note 1

When a new bottle of 10X HBSS is opened, aliquots must be prepared. Otherwise, a precipitate will form. Aliquot size should be small enough so that entire aliquot would be used within ~2 weeks.

Note 2

The number of Assay Plates can be increased. Control A and Control B are run only on the first Assay Plate. All reagent volumes will have to be scaled up accordingly.

Note 3

Test samples must be heat-inactivated prior to test to abrogate any endogenous complement activity. Samples are incubated in a 56^{o}C water bath for 30 minutes. This can be done ahead of the assay and inactivated samples can be re-frozen or stored at 4^{o}C until tested.

Note 4

NICE colony counting software is available at no charge. See www.vaccine.uab.edu for details.

Note 5

Sodium azide is a poison and may be toxic if ingested or absorbed through the skin or eyes. It may react with lead and copper plumbing to form highly explosive metal azides. On disposal of reagents containing sodium azide, flush with a large volume of water to prevent azide build-up or discard in a biohazard bag

Figure 2. LBA plates after color development.