

## SUPPLEMENTAL MATERIAL S1

### OV16 ELISA Protocol

#### A. Reagents.

- PBS 10×: (store at room temperature)
  - NaCl 80 g
  - KCl 2.0 g
  - KH<sub>2</sub>PO<sub>4</sub> 2.4g
  - Na<sub>2</sub>HPO<sub>4</sub> 14.4 g
 Final volume 1L, pH 7.0–7.4
- PBST: (store at room temperature)
  - PBS 1×
  - Tween 20 0.05%
- PBST-BSA (store at 4°C)
  - 1× PBST, Bovine serum albumin 5% (W/V)
- Coating Buffer (store at 4°C)
  - 0.1 M NaHCO<sub>3</sub> (pH 9.6)
- 1M MgCl<sub>2</sub>
- Substrate buffer (store at 4°C in the dark)
  - Diethanolamine 10% (V/V)
  - MgCl<sub>2</sub> 3 mM
  - Adjust pH to 9.8
- 1 mg/mL p-Nitrophenyl phosphate (pNPP) solution (Sigma N-9389)
- Anti-human IgG4 antibody conjugated to biotin (Zymed 05-3840)
- Streptavidin-AP conjugate (Invitrogen 19542018)
- Ov16 GST antigen
- GST
- Positive Control Sera:
  - Constructed of a pool of at least 20 serum samples from patently infected people. Prepare 2-fold serial dilutions starting with 1/20, in PBST-BSA.
- Immunolon 2 HB flat bottomed plates (Thermo Scientific)

#### B. Procedure.

**Create a map: Use the template below. The samples are run in duplicate as indicated.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1:20	STD 1:20	STD 1:5120	STD 1:5120	S5	S9	S13	S17	S21	S25	S29	S33
B	STD 1:40	STD 1:40	STD 1:10240	STD 1:10240	S5	S9	S13	S1	S21	S25	S29	S33
C	STD 1:80	STD 1:80	B	B	S6	S10	S14	S18	S22	S26	S30	S34
D	STD 1:160	STD 1:160	B	B	S6	S10	S14	S18	S22	S26	S30	S34
E	STD 1:320	STD 1:320	S1	S3	S7	S11	S15	S19	S23	S27	S31	S35
F	STD 1:640	STD 1:640	S1	S3	S7	S11	S15	S19	S23	S27	S31	S35
G	STD 1:1280	STD 1:1280	S2	S4	S8	S12	S16	S20	S24	S28	S32	S36
H	STD 1:2560	STD 1:2560	S2	S4	S8	S12	S16	S20	S24	S28	S32	S36

STD = positive control pool dilutions; B = blank wells; S = sample wells.

**2. Elution from paper filter samples:** Punch out duplicate spots from the dried blood samples collected on the filter papers using a standard 6 mm paper punch. Using your map, place the duplicate punches of the blood spots into the sample wells. Add 200 µL of PBST-BSA to each sample. Push the punches to the bottom of the well and then mix 10 times by pipetting. Cover the plates with a plate sealer and incubate them at 4°C overnight. Store the eluted serum samples at –20°C.

**3. Coating plate with antigen:** Dilute Ov16-GST antigen to 2.0 µg/mL in coating buffer. Add 100 µL to each well. Place the plate in a ziplock bag and incubate it overnight at 4°C.

**4. Washing plates:** Wash 4 times with PBST, using a wash bottle. Do not dry between washes. However, dry the plate after last wash.

**5. Blocking plates:** Add 100 µL of PBST-BSA, place the plate in a ziplock bag, and incubate it at 4°C for 1 hour.

**6. Preparation of standard and controls:** During the incubation of step 5 prepare 10 serial 2-fold dilutions of the positive control pool, beginning with a 1:20 dilution.

**7. Empty plate:** After the incubation of step 5, empty the PBST-BSA into the sink and dry the plate. Do not wash.

**8. Adding samples:** Use your map and add 50 µL of each serum sample (positive control dilution, blanks, and eluted samples) to the corresponding wells on the plate map. Place the plate in a ziplock bag and incubate it at room temperature for 2 hours.

**9. Washing plates:** Wash 4 times with PBST, using a wash bottle. Dry the plate after the first wash, and then carry out the remaining three washes without drying the plate between the wash steps. Dry the plate after the 4th wash.

**10. Add conjugate:** 10 minutes before the time is up for the last incubation, prepare the conjugate. Dilute the anti-human IgG4 antibody conjugated to biotin 1:1000 in PBST. Add 50 µL

of the diluted conjugate to all wells. Place the plate in a ziplock bag and incubate it at room temperature for 1 hour.

**11. Washing plates:** Wash 4 times with PBST, using a wash bottle. Do not dry between washes. However, dry the plate after last wash.

**12. Add Streptavidin-AP:** 10 minutes before the time is up for the last incubation, prepare the streptavidin-AP. Dilute streptavidin-AP 1:2000 in PBST. Add 50  $\mu$ L to all wells, cover the plate, and incubate it at room temperature for 1 hour.

**13. Washing plates:** Wash 4 times with PBST, using a wash bottle. Do not dry between washes. However, dry the plate after last wash.

**14. Substrate:** 15 minutes before the incubation period is over switch on the ELISA plate reader. Prepare 1 mg/mL pNPP solution with the substrate buffer. Add 50  $\mu$ L to each well. If your reader reads the whole plate at once, then read the plate until the 1/20 standard is around OD 1.5. If your reader reads well by well, then read only the two wells of the 1/20 standard every minute, and shake the plate between readings. When they are around 0.9 OD, start reading more frequently. When you reach OD 1.1, stop the reaction by adding 25  $\mu$ L of 3 M NaOH to each well. Shake the plates to stop the reaction. Incubate the plate for 5 minutes at room temperature and read it at 405 nm.

### C. Quality assurance.

The standard curve dilutions are assigned the following arbitrary units

Dilution	Units
1:20	2560
1:40	1280
1:80	640
1:160	320
1:320	160
1:640	80
1:1280	40
1:2560	20
1:5120	10
1:10,240	5

The OD values of the individual standard curve wells must fall within the acceptable range of the values shown below\*

Concentration (arbitrary units)	Target Mean (OD)	Acceptable range ( $\pm 2$ standard deviations)
2560	1.55	(1.75, 1.34)
1280	1.46	(1.68, 1.24)
640	1.37	(1.58, 1.16)
320	1.16	(1.41, 0.92)
160	0.77	(1.00, 0.55)
80	0.36	(0.50, 0.23)
40	0.13	(0.19, 0.06)
20	0.04	(0.06, 0.02)
10	0.02	(0.04, 0.00)
5	0.01	(0.02, -0.1)

\*A plot of OD versus units should be linear in the range of 20–160 units, with an  $r^2$  value for the line of  $> 0.95$ . The negative control wells should exhibit an OD value corresponding to  $< 20$  units.

### D. Analysis of results.

The cutoff is set at 40 arbitrary units. Any sample for which both duplicate samples give a reading at or above this point is considered putatively positive. If the sample is putatively positive, repeat the assay, using wells coated with Ov16-GST and GST alone. Samples giving an OD value

$> 40$  units in the confirmatory assay in the Ov16 GST-coated wells but are negative in wells coated with GST alone are confirmed positives.

### SUPPLEMENTAL MATERIAL S2

Calculation of the proportion of a finite population that must be screened to ensure an upper bound of a 95% confidence interval (CI) of a prevalence of  $< 0.1\%$  when all samples are negative.

Sampling small populations represents a special case in which sampling without replacement is carried out on a finite population. In this case, we can define our population of size  $N$  ( $N < \infty$ ) as having a Bernoulli characteristic of variable  $Y$  (e.g.,  $Y = 1$  for antibody positive, or  $Y = 0$  for antibody negative). Because the population is finite, and when sampling this population we conduct a protocol that involves sampling selected individuals once (i.e., sampling without replacement), the distribution of the number of people,  $X$  in a sample of size  $n$  who are antibody positive then follows a hypergeometric distribution

$$P(x|N, M, n) = P(X=x) = \frac{\binom{M}{x} \binom{N-M}{n-x}}{\binom{N}{n}}, L \leq x \leq U, \quad (1.1)$$

where  $M$  is the total number of antibody positive people in the population,  $L = \max(0, M - N + n)$  and  $U = \min(n, M)$ .

Let  $Y_i$  be the  $y$ -value for the  $i$ th person in the population. Then

$$M = \sum_{i=1}^N Y_i,$$

and as previously described,<sup>1</sup> we define

$$\mu = \frac{1}{N} \sum_{i=1}^N Y_i = \frac{M}{N},$$

$$\sigma^2 = \frac{1}{(N-1)} \sum_{i=1}^N (Y_i - \mu)^2,$$

to be the mean and population variance of  $Y_i$ , respectively. In this case, it can be shown that

$$\sigma^2 = \frac{N}{N-1} \mu(1-\mu) = \left( \frac{N}{N-1} \right) \left[ \frac{M}{N} \left( 1 - \frac{M}{N} \right) \right].$$

A sample of size  $n$  is drawn from this population without replacement and  $\mu$  is estimated by

$$\hat{p} = \frac{1}{n} \sum_{i=1}^n Y_i.$$

It is known<sup>1</sup> that

$$\text{Var}(\hat{p}) = \left( \frac{N-n}{N} \right) \frac{\sigma^2}{n}.$$

The quantity  $(N-n)/N$  is called the finite sample correction factor. Because  $\sigma^2$  is unknown in practice, it must be estimated and the usual estimator is the sample estimate

$$s^2 = \frac{1}{n-1} \sum_{i=1}^n (Y_i - \hat{p})^2.$$

It should be noted that the estimator is design unbiased for the ratio  $M/N$ . Because the sample mean is unbiased for the proportion of antibody positive individuals,  $N\hat{p}$  is designed unbiased for  $M$ .

The probability model defined in Equation 1.1 provides the basis for an exact test of the prevalence  $p_0 = M/N$ . We first reparameterize the model by setting  $M = \theta N$  with  $\theta = j/N, j = 0, 1, 2, \dots, N$ , then take the value of  $\hat{j}$  for which  $|p_0 - \frac{\hat{j}}{N}|$  is smallest to be  $M$  in the model. Given the observed number of individuals in the sample who are antibody positive,  $x$ , we then can calculate

$$P(X \leq x | N, M, n) = \sum_{k=L}^x \frac{\binom{M}{k} \binom{N-M}{n-k}}{\binom{N}{n}},$$

where  $L = \max(0, M - N + n)$  and  $X \leq \min(n, M)$ . If this value is less than or equal to a prechosen level (for example  $\alpha = 0.05$ ), we would reject the null hypothesis that the true number of antibody positive individuals in the population is  $M$  in favor of the conclusion that the true value is  $< M$ .

Approximate CIs are often found by means of the application of asymptotic theory based upon the Classical Central Limit Theorem (CLT). If sampling in a finite population is done by random sampling with replacement, the  $n$  observations are then independently and identically distributed, therefore the CLT applies. However, when sampling is done without replacement (such as in a standard serosurvey), the sample observations are not independent and the CLT does not apply. Fortunately, there is a special version of the CLT that applies in this case.<sup>2,3</sup> Agresti and Coull<sup>4</sup> provide a survey of methods for constructing CIs for binomial proportions, a number of which are based on the use of asymptotic theory. In particular, the interval suggested by Wilson<sup>5</sup> leads to an interval that can be applied when no positive individuals are found. The deriva-

tion of the formulas for the end points of the interval are straightforward and lead to the following result:

Let  $N$  be the size of the population,  $n$  be the size of the sample and let

$$k = \sum_{i=1}^n Y_i,$$

be the number of subjects in the sample who are antibody positive. As previously, let  $\hat{p} = k/n$ . The interval then has end points

$$p_{lo} = \frac{\left(\hat{p} + \frac{u^2 f}{n}\right) - u \sqrt{\frac{f}{n} \sqrt{\hat{p}(1-\hat{p}) + \frac{u^2 f}{4n}}}{\left[1 + \frac{u^2 f}{n}\right]},$$

$$p_{hi} = \frac{\left(\hat{p} + \frac{u^2 f}{n}\right) + u \sqrt{\frac{f}{n} \sqrt{\hat{p}(1-\hat{p}) + \frac{u^2 f}{4n}}}{\left[1 + \frac{u^2 f}{n}\right]},$$

where  $f = (N - n) / (N - 1)$  is the finite population correction factor and  $u$  is the appropriate upper tail critical point of the standard normal distribution. For a 95% CI,  $u = 1.96$ . These formulas can also be used to construct one-sided confidence intervals. For example, a 95% upper bound for  $p$  can be calculated by calculating  $p_{hi}$  with  $u = 1.64$ , which leads to the interval  $(0, p_{hi})$  where all of the  $\alpha$  has been put into the upper tail.

## REFERENCES

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