Chapter 38

Ricin-Holotoxin-Based Vaccines: Induction of Potent Ricin-Neutralizing Antibodies

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Abstract

Ricin is one of the most potent and lethal toxins known to which there is no available antidote. Currently, the most promising therapy is based on neutralizing antibodies elicited by active vaccination or given passively. Here, detailed protocols are provided for the production of two ricin holotoxin-based vaccines: monomerized subunit-based vaccine, and a formaldehyde-based ricin toxoid vaccine. Both vaccines were found to be stable with no toxic activity reversion even after long-term storage while eliciting high anti-ricin antibody titers possessing a potent neutralizing activity. The use of these vaccines is highly suitable for both the production of sera that can be used in passive protection experiments and immunization aimed to isolate potent anti-ricin monoclonal antibodies.

Key words Ricin, Holotoxin, Toxoid, Subunit-based vaccine immunization, Neutralizing antibodies, Acetylcholinesterase, HEK293 cells

1 Introduction

Ricin, derived from the plant *Ricinus communis*, consists of two covalently linked subunits: The A-subunit (RTA; Fig. 1) is an *N*-glycosidase that irreversibly inactivates the 28S rRNA of the mammalian 60S ribosome subunit, and the B-subunit (RTB; Fig. 1) is a galactose-specific lectin that mediates the binding of the toxin to the cell membrane [1]. The availability and ease of production and dissemination of the highly toxic ricin render it an attractive tool for bioterrorism and led to the classification of this toxin as a category B select agent by the Center for Disease Control and Prevention (CDC). Currently, there is no available antidote against ricin exposure and to date the most promising anti-ricin therapy is based on neutralizing antibodies elicited by active vaccination or given passively. While RTA-based vaccines can induce the formation of potent ricin-neutralizing antibodies, recent studies have demonstrated that neutralizing epitopes also exist on RTB, suggesting that a holotoxin vaccination should be considered [2–4].

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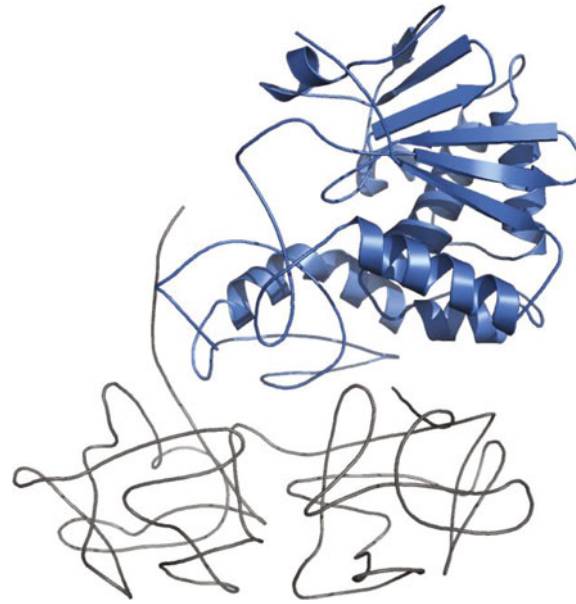

Fig. 1 Crystal structure of ricin holotoxin. Ricin A-subunit (RTA) is colored in *blue* and the B-subunit (RTB) in *gray* (PDB 2AAI)

We and others have found that animals immunized by holotoxin-based vaccines have developed high titers of anti-ricin antibodies that exhibit high affinity and possess excellent neutralization potency [5–9].

In order to inactivate the toxin while retaining its key epitopes needed for efficient immunization, we developed a method by which ricin can be treated with a reducing agent that separates the inter-subunit covalent disulfide bond, thus forming a subunit-based nontoxic vaccine. By adding an alkylation step to this process, we were able to produce a stable and immunogenic preparation that was successfully used for animal immunization.

One of the most direct approaches to inactivate a toxin is to expose it to formaldehyde. Indeed, the resulting ricin-toxoid is a very efficient immunogen that was shown to produce high titers of neutralizing antibodies [5, 10–13]. Use of the ricin-toxoid was limited due to the finding by Griffiths et al. [14] that upon removal of residual formaldehyde from the formulation, the toxic activity returned to the preparation within several days. However, we found that by adopting an earlier version of formaldehyde-based method for inactivation of ricin [15], a very stable vaccine preparation can be made that shows no reversion of ricin toxic activity, even after several months of storage. In addition, we developed a protocol that enables direct vaccination of animals using the native ricin toxin emulsified in adjuvant, a method which was also found to be safe and efficient in the elicitation of high titers of neutralizing antibodies.

Here, we provide detailed protocols for the preparation of ricin-holotoxin-based vaccines by the three different approaches mentioned and the optimal immunization schedules to produce anti-ricin-neutralizing antibodies. We also include a detailed description of our novel sensitive cell-based assay [5], which is used to determine the residual toxicity of ricin in the vaccine preparation and to monitor the production of ricin-neutralizing antibodies throughout the immunization process.

2 Materials

2.1 Equipment

1. Cell culture incubator (37 °C in a humidified 5 % CO_2).
2. 96-Well plate-based colorimeter.
3. General laboratory consumables (1.5 ml micro tubes, 15 and 50 ml centrifuge tubes, cell culture flasks, etc.).
4. Sterile cell culture 96-well plates.
5. ELISA 96-well polystyrene plates with high protein absorption capacity (e.g., Maxisorb, Nunc).
6. Dialysis bags of 10–15 kDa cutoff (alternatively: centrifugal filters with a 10 kDa cutoff).

2.2 Reagents and Buffers

1. Pure ricin in PBS.
2. HEK293-AChE cell line.
3. Dulbecco's modified Eagle medium (DMEM).
4. Tissue culture-grade fetal bovine serum (FBS).
5. Tissue culture-grade 0.05 % trypsin-EDTA.
6. Tissue culture-grade L-glutamine (200 mM).
7. PAT-medium: DMEM supplemented with 10 % AChE-depleted FBS and 2 mM L-glutamine.
8. Dithiothretol (DTT), 1 M.
9. Iodoacetamide 0.5 M (92.5 mg/ml in buffer PBS9): Prepare fresh at the same day and keep covered in aluminum foil at 4 °C.
10. Complete and incomplete Freund's adjuvant.
11. Alkaline-phosphatase-conjugated anti-rabbit antibody.
12. *p*-Nitrophenyl phosphate (PNPP) substrate freshly dissolved in DDW.
13. Benzotonium chloride.
14. ATC/DTNB substrate solution: 0.1 mg/ml BSA, 0.3 mM 5,5′-dithio-bis-(2-nitrobenzoic acid), 50 mM sodium phosphate buffer, pH 8.0, and 0.5 mM acetylthiocholine iodide (ATC).

15. Formaldehyde (37 %).
16. Phosphate-buffer saline (10 mM, pH 7.4. 150 mM NaCl; PBS).
17. Tris–HCl 1 M, pH 8.0.
18. Tris–HCl 1 M, pH 9.0.
19. Buffer PBS9: To 9 ml of PBS add 1 ml of Tris–HCl 1 M pH 9.0. Prepare fresh at the same day and keep at 4 °C.
20. ELISA coating buffer: $NaHCO_3$, 50 mM, pH 9.6.
21. ELISA blocking solution: PBS containing 2 % BSA, 0.05 % Tween-20, and 0.05 % azide.
22. ELISA wash buffer: PBS containing 0.05 % Tween-20.

3 Methods

3.1 Preparation of Ricin Subunit-Based Vaccine

In order to produce a nontoxic vaccine while retaining maximal similarity to the basic structure of ricin, the toxin is treated with a reducing agent in a way that separates the two subunits from each other. To prevent reversion of this process and the reformation of an active toxin, an alkylation step was added to irreversibly modify the free thiols in the vaccine preparation.

1. In a 50 ml tube, prepare 9 ml stock solution of ricin diluted in PBS to a concentration of 1 mg/ml. Pass 100 μl into a 1.5 ml micro tube labeled as "BKG" and keep for further analysis to determine background levels.
2. To the 50 ml tube, slowly add dropwise 1 ml of buffer PBS9 and mix gently.
3. Slowly add dropwise 1.1 ml of DTT 1 M and mix gently.
4. Incubate for 2 h at room temperature. By the end of the incubation period pass 100 μl into a 1.5 ml micro tube, label it as "Ricin-DTT," and keep for further analysis.
5. To the ricin-containing 50 ml tube add slowly 2.75 ml of iodoacetamide 0.5 M and mix gently.
6. Cover the 50 ml tube in aluminum foil and incubate for 2 h at room temperature. At the end of the incubation period pass 100 μl into a 1.5 ml micro tube and label it as "Ricin-IAA."
7. Determine that there are no free non-alkylated thiol residues in the preparation: Prepare three micro tubes, each containing 190 μl of PBS. Add 10 μl from either of the tubes marked as "BKG," "Ricin-DTT," or "Ricin-IAA." To each micro tube add 20 μl of DTNB, mix well, and transfer 100 μl to a 96-well microtiter plate. Measure the optical density in each well at the wavelength of 412 nm while subtracting the reference value read at 650 nm.

Calculate the ratio "Ricin-DTT"/"BKG" (should be >10) and "Ricin-IAA"/"BKG" (should be <2) (*see* **Note 1**).

8. Transfer the mixture into a dialysis bag (10–15 kDa cutoff) and place it in a beaker containing 2 l of PBS. Dialyze for 48 h at 4 °C under constant stirring, changing the PBS reservoir every 12 h.
9. Pass the mixture to a 50 ml tube and centrifuge for 20 min at 17,000 × *g*.
10. Quantify the protein concentration in the supernatant by measuring absorbance at 280 nm. Aliquot in micro tubes and keep at −20 °C.
11. Remove one aliquot to determine residual ricin activity according to the method described under Subheading 3.3 (*see* **Note 2**).

3.2 Preparation of Ricin-Toxoid

Another strategy to produce a safe and effective holotoxin-based vaccine is by inactivating the toxin with formaldehyde. In this protocol, we use a 4.2 % formaldehyde-buffered solution and incubate the mixture at 42–47 °C for extended durations. The resulting preparation was found to be very stable and can be stored for several months without any sign for reversion of ricin toxicity.

1. In a 50 ml tube, prepare 22 ml stock solution of ricin diluted in PBS to a concentration of 2 mg/ml.
2. Freshly dilute the 37 % formaldehyde stock to 8.4 % by mixing 17 ml PBS and 5 ml formaldehyde. Slowly add dropwise 22 ml of the phosphate-buffered formaldehyde to the ricin-containing 50 ml tube (the final concentration of formaldehyde is 4.2 % and that of ricin is 1 mg/ml) (*see* **Note 3**).
3. Incubate the mixture at 47 °C for 18 h.
4. Transfer the mixture to 42 °C and incubate for 30 h.
5. Pour the mixture into a dialysis bag (10–15 kDa cutoff) and place it in a beaker containing 2 l of PBS. Leave for 8 h at 4 °C under constant stirring.
6. Transfer the dialysis bag to a beaker containing 2 l of DDW and continue the dialysis at 4 °C under constant stirring, changing the DDW reservoirevery 12 h.
7. Pass the mixture to a 50 ml tube and centrifuge for 20 min at 17,000 × *g*.
8. Quantify the protein concentration in the supernatant by measuring absorbance at 280 nm.
9. Add benzotonium chloride to a final concentration of 25 μg/ml, aliquot, and store at 4 °C.
10. Determine the residual ricin activity according to the method described under Subheading 3.3 (*see* **Note 2**).

3.3 In Vitro Assessment of Ricin Activity

Residual toxicity in each vaccine preparation should be determined to ascertain that it is safe for immunization. The use of a cell-based assay is advised at this step, since it enables to determine the toxicity of the whole toxin. Here, we provide a detailed protocol for an assay that is based on genetically engineered HEK293-AChE cells that constitutively synthesize and secrete large amounts of acetylcholinesterase (AChE) to the culture medium [16] and any changes in the enzyme level can be sensitively and accurately measured. However, other cell-based assays can be used at this step to determine residual ricin toxicity in the vaccine preparations.

1. HEK293-AChE cells are maintained at 37 °C in a humidified 5 % CO_2 incubator. The cells are cultured in DMEM supplemented with 10 % FBS and 2 mM glutamine and should be subcultured every 3–4 days, upon reaching about 80 % confluence.
2. Detach the cells using trypsin and resuspend in the culture medium to a concentration of 1×10^6 cells/ml in a total volume of 8 ml.
3. Fill six rows of a cell culture 96-well plate by dispensing 100 μl of cell suspension into each well. Rows 1–3 will be used to determine the activity of ricin and rows 4–6 will be used to evaluate the residual activity of the ricin-toxoid preparation.
4. Prepare a 200 μl stock solution of ricin (20 ng/ml) in culture medium in a 1.5 ml micro tube marked 1, and prepare ten micro tubes marked 2–11, each filled with 100 μl of culture medium. Dilute the toxin seriallyby transferring 100 μl from tube #1 to tube #2 and so on to tube #11.
5. Prepare a 200 μl stock solution of 20 μg/ml of detoxified ricin (reduced or toxoid) in culture medium in a 1.5 ml micro tube marked 12, and prepare ten micro tubes marked 13–22, each filled with 100 μl of culture medium. Make serial dilution by transferring 100 μl from tube #12 to the next tube and so on to tube #22 (*see* **Note 4**).
6. Transfer 11 μl from tube #1 to the first three wells in column #1 of the 96-well plate that contains the cell suspension (triplicate points for each tested concentration) and continue accordingly with micro tubes #2 to #11.
7. Repeat the procedure using the samples from tubes 12–22, by transferring the samples into rows 4–6.
8. To column #12, add 11 μl of culture media (this column will be served as the 100 % control).
9. Incubate the 96-well plate at 37 °C in a humidified 5 % CO_2 incubator for 16 h.
10. Replace the culture medium from the three rows of the 96-well plate with fresh 100 μl PAT medium (*see* **Note 5**) and incubate for 2 h.

11. AChE levels are measured in a 96-well microtiter plate. To each well, transfer 11 μl from the 96-well plate and add 100 μl of ATC/DTNB substrate solution. Measure color formation rate in a 96-well plate-based colorimeter that enables kinetic measurements. The optical density in each well should be measured at the wavelength of 412 nm while subtracting the reference value read at 650 nm. The reaction is measured for a period of 5 min, with 30-s intervals, and expressed as V_{max} (mO.D./min) (*see* **Note 6**).
12. Express the AChE levels in each well as percent of control (untreated cells in column 12) and plot the average value of each triplicate as a function of the correspondingricin concentration. Fit the curve using nonlinear regression and determine the concentration needed to reduce levels of secreted AChE to 50 % (IC_{50}).

3.4 Immunization

The following protocol describes the immunization schedules for three ricin-holotoxin-based preparation: native ricin, subunit-based preparation and ricin-toxoid. We found that all three preparations can lead to the elicitation of high anti-ricin titers and to potent neutralizing antibodies. Nevertheless, the epitope profiles recognized by the elicited anti-ricin antibodies (anti-RTA, anti-RTB, and anti-sugar antibodies) in each immunization protocol may vary.

The low toxicity of the subunit-based preparation and of the ricin-toxoid permits an immunization schedule in which a constant and high dose is applied from the start. However, due to the high toxicity of native ricin even when emulsified in Freund's adjuvant, animals are immunized with the native toxin in a stepwise manner with escalating doses of toxin until a minimal level of neutralizing antibodies titer is elicited.

1. Maintain New Zealand white rabbits at 20–22 °C and a relative humidity of 50 ± 10 % on a 12-h light/dark cycle, fed with commercial rodent chow and provided with tap water ad libitum (*see* **Note 7**).
2. To a 15 ml tube, add the desired amount of ricin preparation (either native ricin, subunit preparation, or the ricin-toxoid; *see* **Note 8**) and fill with PBS to a final volume of 2 ml. Add 2 ml of complete Freund's adjuvant and vortex for 2 h at room temperature (*see* **Note 9**).
3. From each rabbit, collect a pre-immune blood sample and then separate and store the serum at −20 °C.
4. Immunize the rabbits by four subcutaneous injections of 250 μl of the ricin-toxoid emulsion given at different sites.
5. Three weeks after the immunization, collect a blood sample and then separate and store the serum at −20 °C.

6. Four weeks after the immunization, boost the animals as described above, except that incomplete Freund's adjuvant is used instead of complete adjuvant.
7. Boost the animals at 1-month intervals (*see* **Note 10**) and collect blood samples 3 weeks after each boost.
8. Coat ELISA 96-well plate with ricin (5 μg/ml in coating buffer) one row for each tested serum sample and one row for backgroundand incubate at room temperature overnight.
9. On the following day, aspirate the ricin-containing buffer, wash the wells three times using wash buffer, add to each well 250 μl of blocking solution, and incubate for 1 h at room temperature.
10. Remove blocking solution and add 90 μl of blocking solution to the first well in each row and 50 μl to the remaining wells.
11. Add 10 μl of the tested serum sample to well #1, mix well, and dilute serially by transferring 50 μl to the next well. Repeat throughout the entire row and discard 50 μl from the wells of column 12. Incubate for 1 h at room temperature.
12. Wash the wells three times using wash buffer and add to each well 50 μl of alkaline-phosphatase-conjugated anti-rabbit antibody. Incubate for 1 h at room temperature.
13. Wash the wells three times, add to each well 50 μl of PNPP substrate, and incubate until color has developed. Read plate at wavelength of 405 nm while subtracting the reference value read at 650 nm (*see* **Note 11**).
14. Plot the optical density as a function of the serum per dilution (Fig. 2a). Fit the curve using nonlinear regression and deter-

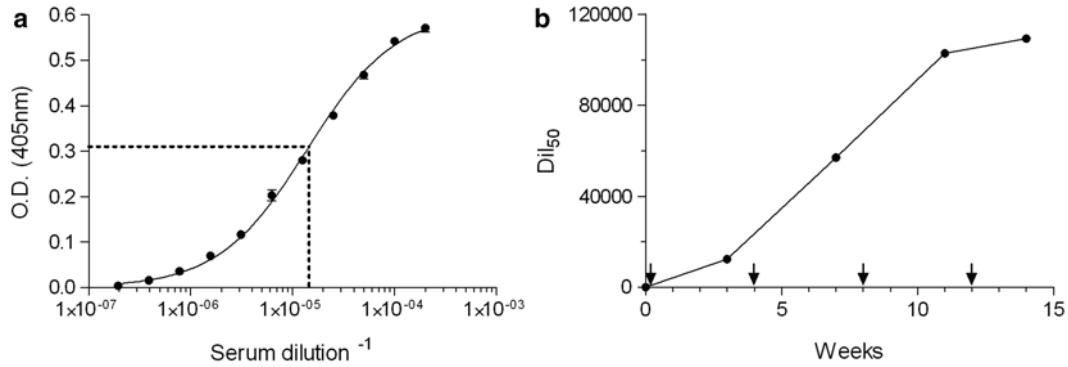

Fig. 2 Serum elicited anti-ricin antibodies following ricin-toxoid immunization. (**a**) Representative ELISA of immunized rabbit anti-ricin serum. *Dashed line* indicates the serum dilution at the 50 % binding point (Dil_{50}). (**b**) Profile of anti-ricin antibody elicitation in rabbit serum following immunization with ricin-toxoid. *Arrows* indicate immunization time points

mine the half dilution value (DIL_{50}) for each serum sample (Fig. 2b) (*see* **Note 12**).

15. Characterize the functional activity of the anti-ricin antibodies using the in vitro neutralization assay (*see* Subheading 3.5) or any other method of choice (*see* **Note 13**).

3.5 In Vitro Neutralization Assay

We utilized the cell-based assay that determines ricin toxicity to determine the formation of ricin-neutralizing antibodies throughout the immunization process. As mentioned above, any cell-based assay that can detect ricin activity can be used at this step. Moreover, in vitro cell-free assays can also be applied in order to determine the specific formation of RTA-neutralizing antibodies.

1. Detach the cells using trypsin and resuspend in the culture medium to a concentration of 1×10^6 cells/ml in a total volume of 4 ml for each tested sample.
2. Dispense 100 μl of cell suspension into the wells of three rows of a 96-well plate.
3. Prepare a 20 ng/ml stock solution of ricin (1.5 ml) in culture medium, and transfer 200 μl to a 1.5 ml micro tube marked 1, and 100 μl to 9 additional micro tubes marked 2–10.
4. To tube #1, add 22 μl of the tested rabbit sera, mix well, and dilute serially by transferring 100 μl from tube #1 to tube #2 and so on.
5. Transfer 11 μl from tube #1 to three wells in column #1 of the 96-well plate that contains the cell suspension (triplicate points for each antibody dilution) and continue accordingly with the other micro tubes.
6. To column #11 add 11 μl from the ricin stock solution (positive control) and to column #12 add 11 μl of culture media (100 % control).
7. Incubate the 96-well plate at 37 °C in a humidified 5 % CO_2 incubator for 16 h.
8. Replace the culture medium from the three rows of the 96-well plate with fresh 100 μl PAT medium and incubate for 2 h.
9. Measure AChE levels in each well (as described under Subheading 3.3), express it as percent of control (untreated cells in column 12), and plot the average value of each triplicate as a function of the serum per dilution (Fig. 3a). Fit the curve using nonlinear regression and determine the effective dilution of serum needed to neutralize 50 % (ED_{50}) of the toxin (*see* **Note 14**).
10. Continue with monthly booster injections until the ED_{50} value reaches plateau (Fig. 3b).

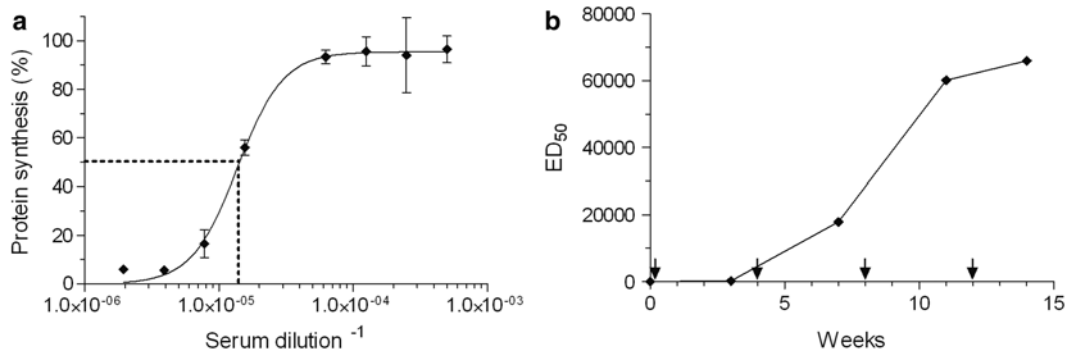

Fig. 3 In vitro neutralization assay. (**a**) Representative profile of ricin neutralization by sera of ricin-toxoid-immunized rabbit. *Dashed line* indicates the serum dilution needed to inhibit 50 % of ricin activity (ED_{50}). (**b**) Profile of ricin-neutralizing antibodies elicited in rabbit serum following immunization with ricin-toxoid. *Arrows* indicate immunization time points

4 Notes

1. Efficient alkylation of free thiols is necessary for irreversible monomerization of the toxin subunits in order to produce a safe and stable vaccine preparation. Therefore, if the ratio of "Ricin-IAA"/"BKG" is >2, either repeat **steps 5** and **6** at a longer incubation period or use a freshly prepared iodoacetamide stock solution.
2. The residual ricin activity in the preparations should be less than 1 %; otherwise it may be toxic to the immunized animals.
3. Extra care should be paid at this point as adding formaldehyde too fast can precipitate the protein. Gentle vortexing can be applied throughout this step.
4. It is expected that the residual ricin activity in the preparations will be less than 1% of that of the original stock and therefore the stock concentration of the ricin-toxoid is 1000 times higher than the ricin stock. If a linear curve is not generated then this step should be repeated using higher or lower stock concentrations, accordingly.
5. To reduce the background signal arising from cholinesterases originated from the supplemented FBS, cholinesterase-depleted FBS is used. However, the culture medium can be replaced with fresh DMEM and the background should be properly subtracted from all reading.
6. Typically, the AChE levels in the control untreated cells are in the range of 200–300 mO.D./min. The enzyme concentration can be measured accurately up to 300 mO.D./min. If a higher

value is reached then a smaller volume of the culture medium should be taken (e.g., 5 μl from the tested wells and 6 μl of fresh medium). A much lower value may imply that improper amount of cells were seeded and the test should be repeated.

7. An approval should be obtained from an ethic committee before the beginning of the immunization, according to local regulations.
8. The doses for primary immunization are native toxin—1 μg; subunit preparation—60 μg; and ricin-toxoid—100 μg.
9. Since a significant amount of the formed homogenous solution cannot be recovered from the tube wall, we double the starting volume.
10. The boost doses for the native toxin are 4, 25, and 100 μg. The subunit preparation and the ricin-toxoid boosts are administered at 60 μg and 100 μg, respectively, throughout the immunization schedule.
11. In order to produce a reliable and accurate curve, the color development should be monitored carefully so that measurement would be performed when the absorbance in the wells containing the lowest serum dilution is in the range of 0.5–0.8 O.D. Above that point the rate of color development is not linear and therefore will affect the interpretations of the results.
12. We found that monitoring the immunization process by determining the DIL_{50} in each time point is much more accurate than the classic endpoint titer. The major drawback of the endpoint titer assay is that duplicates can vary significantly (by a factor of 2–4) and require the use of geometrical mean, etc. Therefore, changes in the antibody titer between consecutive boosts that are less than twofold might be unnoticed and will lead to premature cessation of the immunization process. However, the DIL_{50} value is a continuous number that allows good replicates (with deviations of usually <15 %) and therefore better reflects the immunization status.
13. Other methods to assess the quality of the elicited anti-ricin antibodies that may apply at this stage are among others measuring the apparent affinity toward ricin (using BIAcore, Octet, etc.); determining functional affinity (using a chaotrophic agent such as KSCN); and determining the relative distribution of antibodies that recognize each of the two subunits of ricin or the sugar moieties and performing an *in vivo* protection assay.
14. It is important to make sure that the positive control (ricin without serum) leads to 90–95 % inhibition and that a full curve is obtained; otherwise start from higher sera dilution.

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