

# Journal of Visualized Experiments

## Optimized Interferon-gamma ELISpot assay to measure T cell responses in the guinea pig model after vaccination --Manuscript Draft--

<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE58595R1
<b>Full Title:</b>	Optimized Interferon-gamma ELISpot assay to measure T cell responses in the guinea pig model after vaccination
<b>Keywords:</b>	Enzyme-linked immune Spot Assay; Guinea Pigs; T-lymphocytes; Vaccination; Interferon-gamma; Administration, Cutaneous
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<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
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San Diego, May 30<sup>th</sup> 2018

Dear Dr. Upponi,

Please find enclosed our manuscript entitled "Optimized Interferon-gamma ELISpot assay to measure T cell responses in the guinea pig model after vaccination" that we would like to be considered for publication in Journal of Visualized Experiments.

This paper highlights a protocol for an optimized ELISpot assay specific for the guinea pig model. Our protocol describes the processing of peripheral blood samples to obtain PBMCs. We give detailed instructions for preparing assay plates and testing the processed PBMCs. The manuscript provides representative results regarding number and viability of PBMCs and images of plate wells with Interferon-gamma positive spot to inform the reader about expected outcome when applying our protocol. We consider of value publishing these data in Journal of Visualized Experiments, as they can be utilized to detect antigen-specific T-cell responses in the guinea pig animal model. Guinea pig is a highly relevant model for assessing many biologics and modeling disease, however it has been neglected due to the paucity of assay reagents and protocols available. For example, the physiology of the guinea pig skin is similar to that of humans, and thus is a highly relevant model for the development of therapeutics and vaccines which are delivered to the skin. The described IFN-g ELISpot is currently employed in our laboratory to successfully model vaccine T cell responses.

The techniques presented in this paper and demonstrated in video format will be highly useful for researchers working in the field of vaccine development and skin-delivery technologies.

Katherine Schultheis, Hubert Schaefer and Trevor R.F. Smith designed the procedures described in the manuscript. Katherine Schultheis, Holly M. Pugh, Bryan S. Yung, Janet Oh and Jacklyn Nguyen performed the experiments and analyzed the data. Finally, Katherine Schultheis, Trevor R.F. Smith, Kate E. Broderick and Laurent Humeau wrote the manuscript.

Thank you for your consideration of this manuscript. We look forward to hearing from you.

Sincerely yours,

Katherine Schultheis

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**TITLE:**

Optimized Interferon-gamma ELISpot Assay to Measure T Cell Responses in the Guinea Pig Model After Vaccination

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**KEYWORDS:**

Enzyme-linked immune spot assay, guinea pigs, T-lymphocytes, vaccination, interferon-gamma, administration, cutaneous

**SUMMARY:**

The development of the interferon-gamma ELISpot assay for guinea pig PBMCs allows the characterization of T-cell responses in this highly relevant model for studying infectious diseases. We have applied the assay to measure T cell responses associated with intradermal delivery of DNA vaccines.

**ABSTRACT:**

The guinea pig has played a pivotal role as a relevant small animal model in the development of vaccines for infectious diseases such as tuberculosis, influenza, diphtheria, and viral hemorrhagic fevers. We have demonstrated that plasmid-DNA (pDNA) vaccine delivery into the skin elicits robust humoral responses in the guinea pig. However, the use of this animal to model immune responses was somewhat limited in the past due to the lack of available reagents and protocols to study T cell responses. T cells play a pivotal role in both immunoprophylactic and immunotherapeutic mechanisms. Understanding T cell responses is crucial for the development of infectious disease and oncology vaccines and accommodating delivery devices. Here we describe an interferon-gamma (IFN- $\gamma$ ) enzyme-linked immunospot (ELISpot) assay for guinea pig

peripheral blood mononuclear cells (PBMCs). The assay enables researchers to characterize vaccine-specific T-cell responses in this important rodent model. The ability to assay cells isolated from the peripheral blood provides the opportunity to track immunogenicity in individual animals.

## **INTRODUCTION:**

The protocol described here permits the detection of interferon-gamma (IFN- $\gamma$ ) secreting cells after antigen recall in a peripheral blood mononuclear cell (PBMC) population harvested from Hartley guinea pigs. We have applied the assay to characterize the kinetics and magnitudes of antigen-specific T cell responses to an Influenza vaccination-regimen in the guinea pig. We believe this protocol will significantly propel the pre-clinical development of vaccination programs in this highly relevant animal model.

T cells elicited by vaccines play an essential role in the protection against infectious agents and immunotherapeutic pathways associated with other diseases. The importance of T cells has been highlighted in multiple vaccine studies. Immunization of ferrets and mice with a plasmid DNA (pDNA) encoding for H5 hemagglutinin and N1 neuraminidase provided protection from morbidity and mortality in an influenza virus challenge in the absence of neutralizing antibodies, indicating the importance of T cell immunity<sup>1</sup>. In addition to strong neutralizing humoral response, T-cells play a crucial role for not only viral clearance<sup>2</sup> but also protection from infection with respiratory syncytial virus (RSV) in mice<sup>3</sup>. In humans, pre-existing CD8<sup>+</sup> T cells were associated with decreased disease severity during the H1N1 pandemic in 2009<sup>4</sup>. CD4<sup>+</sup> T cell counts together with certain cytokine plasma concentration are correlated with disease severity in RSV-infected children<sup>5</sup>.

The guinea pig has gained prominence as a laboratory model for research and development in various areas of medicine such as skin sensitization, nutritional research, studies of the auditory system and, most relevant for this work, infectious diseases. It was crucial for the discovery of vaccines against tuberculosis and diphtheria. More recently the guinea pig is used as a model for Influenza<sup>6</sup> and Ebola<sup>7</sup>. Furthermore, possessing physiological similarities to human skin<sup>8</sup>, the guinea pig offers an accessible small animal model for dermal drug delivery methods. In contrast to its importance as a laboratory model, the availability of guinea pig specific assays and probes to characterize immune responses remains limited<sup>9</sup>. Basic cellular assays such as the IFN- $\gamma$  enzyme-linked immunospot (ELISpot-assay) that is routinely used in pre-clinical and clinical research to enumerate T cell responses have not been available.

The first solid-phase enzyme-linked immunospot assays were used to determine the number of specific antibody-secreting cells in a diverse B cell-population<sup>10,11</sup>. The format has advanced to detect cells secreting cytokines including IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, GM-CSF, TNF-alpha, TNF-beta, granzyme B, and IFN- $\gamma$ . The ELISpot assay possesses high sensitivity; potentially each cytokine-producing cell can be detected. The limit of detection for ELISpot assays has been reported to be lower than 10 spots per 100000 PBMCs<sup>12</sup>. Recently a guinea-pig IFN- $\gamma$  specific antibody pair was made available<sup>13</sup>, and this has opened up the opportunity to address this deficiency in the field.

IFN- $\gamma$  is considered an important effector molecule in the adaptive immune response; it can be produced and secreted by both CD4 and CD8 T cells upon activation. IFN- $\gamma$  has a wide range of biological functions in the context of an immune response to a virus infection, such as the activation of macrophages and the up regulation of major histocompatibility complex (MHC) I and MHCII expression. It also promotes B-cell differentiation, inhibits T-helper-2 cell growth, and activates natural killer cells. IFN- $\gamma$  blocks viral replication in infected somatic cells and upregulates expression of MHC molecules. Thus, the production of IFN- $\gamma$  is a very important indication of the quality of the T cell response to a vaccine or pathogen.

An important aspect of the here presented IFN- $\gamma$  ELISpot is the use of PBMCs rather than splenocytes<sup>13</sup>. PBMCs can be obtained by processing a non-terminal collected blood sample, whereas the collection of splenocytes requires animal euthanization before harvesting of the spleen. The use of PBMCs enables IFN- $\gamma$  T-cell responses to be monitored over a period of time and evaluation of the effects on the T-cell responses such as prime-boost regimens<sup>14</sup>.

For researchers who are currently using the guinea pig as an animal model, the IFN- $\gamma$  ELISpot method will broaden the range of scientific data they can gain. Its availability may now reduce the necessity to conduct studies with less relevant animal models for the target disease, which were previously chosen due to reagent availability for examination of cellular responses. The use of PBMCs rather than spleen or lymph nodes allows for non-terminal experiments and continuous monitoring of individual animals.

Below we provide a detailed description of the steps involved in the detection of an IFN- $\gamma$  cellular response in guinea pigs to pDNA vaccine. We outline our specific delivery procedure and describe the general ELISpot assay encompassing blood sampling, blood processing, PBMC harvest, assay procedure, and data analysis. A schematic of the ELISpot assay is depicted in **Figure 1**.

[Place **Figure 1** here]

## **PROTOCOL:**

All methods described here have been approved by the Animal Care and Use Committee (ACUC) of Acculab.

Note: The protocol requires the use of potential hazardous materials. Please refer to manufacturer's MSDS and wear proper personal equipment (PPE) throughout the procedure.

### **1. Preparation of the Guinea-pig Immunization Site, Intradermal Mantoux-injection, and Electroporation Procedure**

1.1. Prepare animal and treatment site for injection and electroporation (EP) procedure.

1.1.1. Place guinea pig (Hartley, female, 12 weeks of age) in an induction chamber and anaesthetize with 5% Isoflurane-vapor.

1.1.2. Confirm sufficient depth of anesthesia by testing for absence of withdrawal reflexes in response to noxious stimuli like toe or ear pinch.

1.1.3. Remove animal from the chamber and place nose cone in position, providing 2% Isoflurane vapor to maintain anesthesia throughout the procedure.

1.1.4. Shave area of approximately 4 cm<sup>2</sup> on abdominal flank.

1.1.5. Disinfect shaved area with ethanol swab.

1.2. Inject pDNA-formulation and perform electroporation procedure.

1.2.1. Use an insulin syringe to inject 100 µL of formulation into the dermis by the Mantoux technique.

1.2.2. Insert the needle in a bevel-up orientation at a 10° angle with the animal's body and inject the formulation. For detailed instructions, review part one of the CDC's Mantoux Tuberculin skin testing facilitator guide<sup>15</sup>.

1.2.3. Remove the needle and discard into a sharps container and immediately apply electrical field across the injection-wheal using an intradermal (ID) electroporation device.

1.2.4. Remove animal from anesthesia and monitor its recovery which can be considered complete when the animal fully regains its righting reflex (*i.e.*, when it is able to hold itself in its normal upright position).

## **2. Non-terminal Bleeding**

2.1. Perform steps 4.1.1-4.1.6 the day before taking blood samples to allow for the required assay plate incubation time in step 4.1.6. See **Figure 1** for protocol outline.

2.2. Place guinea pig in an induction chamber and anaesthetize animal as described in 1.1.1-1.1.3.

2.3. Using a 3 mL syringe with a 27G, ½ inch needle, punctuate the jugular vein of the anaesthetized animal as previously described<sup>16</sup> and collect 3 mL of blood.

2.4. To avoid blood clotting, immediately transfer blood into K<sub>2</sub>EDTA blood collection tube, invert tube several times to mix blood with anti-coagulant, and place on ice.

2.5. Keep blood on ice until blood processing (step 3.1.2).

2.6. Monitor animal's recovery as described in step 1.2.4.

### 3. Blood Sample Processing

3.1. Separate blood components by density-gradient centrifugation.

3.1.1. Perform steps 3.1.2 through 4.2.3 in a biosafety cabinet applying aseptic technique to avoid contamination.

3.1.2. Dilute blood 1:1 by mixing with equal volume of Hank's balanced salt solution (HBSS).

3.1.3. Bring density-gradient medium to room temperature. Add 4.5 mL of density-gradient medium in a 15 mL conical tube. Layer diluted blood gradually over density-gradient medium. Avoid disturbance of the interface.

3.1.4. Centrifuge tubes at 800 x g for 30 min with brake off.

Note: As an alternative, specialized PBMC-isolation devices can be used for PBMC-separation. HBSS-diluted blood is added to 15 mL PBMC-isolation device filled with 3.5 mL of density-gradient medium and then centrifuged at 1200 x g for 10 min (brake on).

3.2. Harvest PBMCs from buffy coat layer.

3.2.1. Cautiously remove tubes from centrifuge and avoid disturbance of the layers. Do not pour off content. Refer to **Figure 2** to correctly identify the layers after density-gradient centrifugation.

3.2.2. Harvest PBMCs by aspirating the complete buffy-coat layer. Avoid aspirating density gradient medium below the buffy-coat layer.

3.2.3. Transfer harvested cells to a new 15 mL tube. Dilute harvested cells in R10 medium [10% (v/v) heat-deactivated fetal bovine serum (FBS) and 1% (v/v) pen/strep in RPMI1640 medium] to a total volume of 15 mL.

3.2.4. Pellet cells by centrifuging at 450 x g for 5 min. Discard supernatant and re-suspend cells in 15 mL of R10 medium to wash cells.

3.2.5. Repeat step 3.2.4. Discard supernatant from last washing step and re-suspend cells in 1 mL of R10 medium.

3.2.6. Pass 1 mL cell-suspension through 70 µm cell-strainer into a new tube.

3.2.7. Count cells and determine number of live cells by the trypan-blue exclusion test<sup>17</sup>. Dilute cells with R10 medium to a concentration of  $1 \times 10^6$  live cells per mL.

#### 4. Preparation of ELISpot-plates and Cell-stimulation

4.1. Coat and block wells of a 96-well ELISpot polyvinylidene fluoride (PVDF)-membrane plate before seeding the PBMCs.

4.1.1. Add 15  $\mu\text{L}$  of 35% ethanol per well to ELISpot assay plates. Take extra care to ensure that the complete membrane comes into contact with ethanol.

Note: Ethanol pretreatment is to be done at room temperature (RT).

4.1.2. After 60 s, add 150  $\mu\text{L}$  of 1x phosphate-buffered saline (PBS) per well and then empty out ethanol/PBS by inverting the plates to stop ethanol-pretreatment. Do not incubate the well-membrane longer than 60 s.

4.1.3. Do not let membrane dry out during the following steps of the procedure.

4.1.4. To wash the plates, add 250  $\mu\text{L}$  of 1x PBS per well. Remove PBS by inverting the plates.

4.1.5. Repeat step 4.1.4 two more times. Add 100  $\mu\text{L}$ /well of 5  $\mu\text{g}/\text{mL}$  capture anti-guinea pig IFN- $\gamma$  antibody V-E4 in PBS to coat the plates.

4.1.6. Incubate plates with coating solution for at least 12 h at 4  $^{\circ}\text{C}$ .

4.1.7. Wash plates by repeating step 4.1.4 three times.

4.1.8. Add 200  $\mu\text{L}$ /well blocking buffer [10%(w/v) sucrose and 2%(w/v) bovine serum albumin (BSA) in PBS] and incubate for 2 h at RT. Wash plates by repeating step 4.1.4 three times.

4.2. Plate PBMCs with antigen-specific peptides, positive and negative controls.

4.2.1. Include triplicates of positive control, negative control, and each experiment-specific stimulant (*e.g.*, protein-peptide pool) for each PBMC sample in the plate template. Dilute protein-peptide pool in R10 medium to 3x desired final peptide concentration.

4.2.2. Add 50  $\mu\text{L}$  of peptide-R10 medium to respective wells indicated for stimulant-testing on plate template. Add 50  $\mu\text{L}$  of empty peptide formulation [*e.g.*, dimethyl sulfoxide (DMSO)] in R10 medium into respective wells indicated for negative control on plate template. Add 5  $\mu\text{g}/\text{mL}$  concanavalin A (ConA) in R10 medium into respective wells indicated for positive control on plate template.

4.2.3. Add 100  $\mu\text{L}$  of PBMC cell-suspension to all wells.

Note: Add medium with stimulants to the ELISpot plates first and the PBMC suspension second.



4.2.4. Place ELISpot plates on an even surface/rack in the incubator. Incubate plates in humidified 5% CO<sub>2</sub> atmosphere at 37 °C for 18 h and avoid any disturbance of the plates during the incubation time.

## 5. Detection of Interferon-gamma Positive Spots

Note: Steps in this section do not require aseptic technique.

5.1. Carefully remove plates from the incubator and remove supernatant from wells by inverting the plates. Wash Plates by repeating step 4.1.4 three times.

5.2. Add 100 µL/well of 2 µg/mL biotinylated detection anti-guinea pig IFN-γ antibody N-G3 diluted in blocking buffer. Filter (0.22 µm pore-size filter) antibody solution before use to reduce background.

5.3. Incubate with detection antibody for 2 h at RT.

5.4. Discard antibody-solution by inverting the plate and wash by repeating step 4.1.4 three times. Discard PBS from last washing step by inverting the plate.

5.5. Add 100 µL/well of alkaline phosphatase (ALP)-conjugated streptavidin diluted in blocking buffer. Filter (0.22 µm filter) solution before use to reduce background.

5.6. Incubate with ALP-streptavidin conjugate for 1 h at RT.

5.7. Discard ALP-streptavidin solution by inverting the plates. Wash plates by repeating step 4.1.4. Remove excess PBS by inverting the plate and blotting it against a clean paper towel.

5.8. Wash plates by adding 250 µL/well ultra-pure deionized (DI) water. Remove water by inverting the plates and remove excess water blotting inverted plate against a clean paper towel.

5.9. Add 100 µL of 5-bromo-4-chloro-3-indoloyl phosphate-nitro blue tetrazolium (BCIP/NBT) substrate solution into each well. Incubate for 20 min at RT protected from light.

CAUTION: BCIP/NBT is highly flammable and toxic if swallowed, in contact with skin, or inhaled. Refer to the MSDS before use.

5.10. Rinse the plate 4 times with DI water by adding DI water with a pipette to the wells of the plate or by rinsing the entire plate with running DI water from a laboratory faucet.

5.11. Invert plate and tap to remove excess water. Remove plastic drainage from bottom of ELISpot plates and allow the membranes to completely dry.

5.12. Quantify spots manually or by using an automated ELISpot reader equipped with image analysis software.

#### **REPRESENTATIVE RESULTS:**

The results presented here serve as a reference for expected outcomes following the use of this protocol, emphasizing the importance of crucial steps and confirming the benefits of described optimizations.

After density-gradient centrifugation, as described in step 3.1 of the protocol, the red viscous liquid at the bottom of the tube will contain most of the red blood cells. As shown in **Figure 2A**, above the red blood cells is a layer of the density-medium. Between a layer of clear or yellow plasma on top and the density gradient medium is the white or light brown buffy coat layer, which contains most of the white blood cells and platelets. Incomplete separation would manifest as the presence of red blood cells on top of the density-gradient medium. The red coloration of the plasma in **Figure 2A** is most probably due to hemolysis, as this blood sample was stored in the collection tube 1.5 h before being processed. **Figure 2B** shows the gradient before (left) and after (right) centrifugation in PBMC-isolation device. Blood sample in **Figure 2B** was processed with minimal delay after blood collection, and the plasma coloration is yellow.

The effect of pre-wetting the membranes with ethanol (see step 4.1 of the protocol) on spot-development is shown in **Figure 3**. Spots in pre-treated wells display improved definition, and there is a reduction in the number of spots in medium/DMSO negative control wells (**Figure 3A**). Typical viability of processed guinea pig PBMCs ranges around 90% and is similar for both regular 15 mL tubes and PBMC-isolation devices (**Figure 3B**). Yield of viable cells from a 3 mL blood sample typically ranges between  $3 \times 10^6$  and  $5 \times 10^6$ .

Animals were immunized with plasmid DNA encoding the nucleoprotein (NP) of H1N1 Influenza strain A/PuertoRico8. **Figure 4** shows enumeration of the T-cell IFN- $\gamma$  responses presented as spot-forming units (SFU) generated across the duration of a vaccination regimen. ELISpot assay with PBMCs harvested from non-immunized animals results in negligible spot counts (no tx). Fourteen days after the first immunization (prime), an average of 970 IFN- $\gamma$  spots per million PBMCs were counted after immunogenic stimulation. Seven days after the second immunization (boost), T-cell responses against all three pools were expanded, reaching an average of 5020 IFN- $\gamma$  SFUs/ $10^6$  PBMCs. Forty-six days after the second immunization (memory), an average total of 6310 IFN- $\gamma$  SFUs/ $10^6$  PBMCs were counted in peripheral blood.

#### **FIGURE LEGENDS:**

**Figure 1: Schematic overview of the guinea pig ELISpot protocol.**

**Figure 2: Representative images of layers after density-gradient centrifugation. (A)** Blood sample before (left) and after (right) centrifugation in regular tubes. **(B)** Blood sample before (left) and after (right) centrifugation in PBMC-isolation devices.

**Figure 3: Optimization of the protocol.** (A) Typical appearance of IFN- $\gamma$  positive spots in an ELISpot assay plate well developed according to the protocol. Example triplicate wells are shown after pre-treatment with ethanol (right) or without pre-treatment (left). Cells were incubated overnight either with DMSO (top) as no-stimulus control, antigen-matched peptides (middle), or the mitogen ConA (bottom). PBMCs originated from the same individual animal. Wells were imaged using an automated plate-scanner. (B) Comparison of different density-gradient tubes on processed cells. 3 mL of blood samples from 3 individual guinea pigs were collected. 1.5 mL of each sample were processed using either regular tubes (density gradient medium) or PBMC-isolation devices. Viability (left, %  $\pm$  SEM) and number of live cells (right,  $\times 10^6 \pm$  SEM) were determined using an automatic cell counting system and trypan-blue exclusion assay.

**Figure 4: Detection of robust cellular immune responses over the course of a vaccination regimen.** On days 1 and 15, 30  $\mu$ g of pDNA encoding influenza A nucleoprotein (NP) was delivered intra-dermally to abdominal flank of Hartley guinea pigs immediately followed by skin-electroporation. IFN- $\gamma$  ELISpot response was measured 14 days after the first immunization (prime), and 7 days (boost) and 46 days (memory) after the second immunization. Mean SFUs + SEM were plotted for 5 treated guinea pigs and 2 untreated guinea pigs (no tx).

## DISCUSSION:

The guinea pig is a valuable animal model for the pre-clinical development of vaccines and intradermal delivery strategies. The above protocol describes the methodology to measure antigen-specific T cell responses in this highly relevant model. The assay provides a clear enumeration of peripheral T-cells producing Interferon-gamma upon stimulation with antigen-specific peptides. The kinetics of the immune response can be monitored by non-terminal blood sampling.

We optimized the protocol and identified critical aspects to obtain optimal results using this assay. For example, the formation of blood clots in the collection tube will result in suboptimal PBMC recovery and viability. Guinea pig blood clots very rapidly<sup>18</sup>. It's crucial to perform the blood draw quickly. Au-Birck *et al.* provide a comprehensive guide for bleeding techniques in the guinea-pig model<sup>16</sup>. Since the blood collection requires general anesthesia, it is recommended to review applicable standard operating procedures of this aspect of the procedure<sup>19</sup>. Insufficiently anaesthetized guinea pigs will react by moving their legs or vocalization after a brief pinch of the tissue between their toes with your fingernails or by flinching their ears and moving their whiskers forward after pinching their ear. The immediate transfer of the blood into a tube with anticoagulant and mix thoroughly by rolling or inverting the tube several times is an essential step in this protocol. For the here described protocol EDTA-tubes were used, and no other anticoagulants were tested. Please note that EDTA is hypertonic, tubes should ideally be filled more than half full, and therefore the appropriate tube size for the sample volume must be used.

When performed correctly, density gradient centrifugation consistently results in clean PBMC preparation. The density-gradient medium must be at room temperature when layering the gradient before centrifugation, and brakes should not be used for stopping the centrifuge when using regular tubes. A significant improvement in terms of practicality of the PBMC-processing

was the combination of density gradient centrifugation with PBMC-isolation devices. This allows for a reduction in centrifugation time. Density gradient centrifugation in PBMC-isolation devices and regular tubes results in similar viability, live cell counts of processed PBMCs, and spot formation. Independent of the type of tube used for the separation, the buffy coat harvest should follow immediately. Over an extended period of time contact with the density gradient medium is cytotoxic to the PBMCs.

Accurate counting of viable PBMCs is important to seed consistently equal numbers of cells into the assay-plate wells. Some method of live/dead discrimination should be applied. In our lab, we use the trypan-blue exclusion assay and an automated cell-counter.

Spot quality, defined as sharp-edged and high contrasted spots, will result in improved accuracy and consistent spot counting, especially when using an automated counting system. In line with the manufacturer's recommendations, we observed ethanol pre-treatments of the ELISpot plates to be a crucial step to reduce the number of background spots and improve the definition of the spots. The use of a plate reader to image the ELISpot assay-plates at the end of the protocol is highly recommended. Original images of each well can be easily and efficiently obtained and stored. Using an image analysis software digital images also allow for objective analysis and spot counting compared to manual counting by individual operators.

An absolute necessity for the development of the assay described here was the generation of a suitable anti-guinea pig Interferon-gamma antibody pair. Mouse monoclonal antibodies V-E4 and N-G3 were developed by hybridoma-technique with B-cell clones from mice which were immunized with recombinant guinea-pig interferon gamma<sup>20</sup>. V-E4, which is IgG1 isotype, and N-G3, which is IgG2a, are reported to bind both to the recombinant and native antigen. Assigning V-E4 as the capture antibody and biotinylated N-G3 as the detection antibody resulted in high sensitivity for both recombinant and native protein while retaining low background signal in a sandwich-ELISA format. Both antibodies are available to the scientific community through a manufacturing agreement led by the laboratory of Dr. Hubert Schaefer, who is co-author of this publication. Requests will be received by Dr. Schaefer's lab and then manufactured by a commercial partner.

Interferon-gamma is reported to be unstable in regular buffers or media<sup>21</sup>. Schaefer *et al.*<sup>20</sup> reported only a moderate decrease of recovery rate when using degraded IFN- $\gamma$ , which had lost biological functionality, in an ELISA format using antibodies V-E4 and N-G3. However, increasing the incubation time of peptide stimulation of PBMCs over 18 h should be carefully tested.

The advantages of using PBMCs has been discussed. However, the here described assay only reflects antigen-specific responses of circulating T-cells in the periphery. Tissue-infiltrating T-cells or cells isolated from lymphatic organs, such as spleen and lymph nodes, may exhibit different properties<sup>22-24</sup>. No significant differences in cellular responses were observed upon comparison of interferon-gamma spots from PBMCs and splenocytes from guinea pigs that were immunized with the same pNP influenza vaccine<sup>14</sup>.

In this protocol, we described an intradermal vaccination procedure. A principal rationale for ID immunization is targeting the high density of dendritic cells present in the skin<sup>25</sup>. We and others have shown that these cells can be specifically targeted by adapting delivery-method<sup>26</sup>, formulation<sup>27</sup>, or drug-design<sup>28</sup>. Activated professional antigen-presenting cells may migrate to a draining lymph node and activate the adaptive immune system<sup>29-32</sup>. Dendritic cells are the essential antigen presentation cell-type for priming productive cellular immune responses.

For this study animals were immunized with plasmid DNA encoding the nucleoprotein of influenza H1N1 strain A/PuertoRico/8. Skin delivery of this pDNA vaccine (pNP) in combination with electroporation had been shown to elicit antigen-specific humoral responses in guinea pigs<sup>33</sup>, ferrets, and non-human primates (NHPs)<sup>1,34</sup>. Additionally, this vaccine elicited robust T-cell responses in mice after delivery into the epidermis<sup>35</sup>, which could be attributed to CD4 and CD8 T-cells by stimulating with peptides representing specific epitopes for these cell-populations. The pNP vaccine was also immunogenic after mucosal delivery as demonstrated by generation of humoral responses in rabbits and guinea pigs, as well as cellular and humoral responses in mice<sup>36</sup>. Most recently, our group was able to demonstrate the generation of IFN- $\gamma$ -T-cell responses in the rabbit after intra-muscular delivery (unpublished data).

Currently, the observed Interferon-gamma spots in the guinea pig ELISpot cannot be allocated to a CD4+ or CD8+ T-cell subset. Especially for the design and development of immune therapies targeting the skin, it would be very useful to determine whether observed interferon-gamma spot frequencies are caused by expansion of one particular subset or a balanced response of both in order to evaluate the effectiveness of the vaccination to trigger cross-presentation. This limitation might be overcome by negative cell-sorting for CD4 or CD8 prior to seeding the cells on the plate or by identification of MHC class II (for CD4 responses) or MHC class I (for CD8 responses) restricted epitopes.

The here described Interferon-gamma ELISpot assay using guinea pig PBMCs addresses the need to assess the course of cellular responses in the guinea pig laboratory model. This will refine the development of vaccines and skin delivery protocols. We believe that it allows for the employment of this relevant animal model to study diseases with important T-cell components such as TB<sup>37</sup>, Ebola<sup>38</sup>, HSV<sup>39</sup>, and others. It will reduce the use of less relevant animal models.

#### **ACKNOWLEDGMENTS:**

We would like to thank the members of the Inovio Pharmaceuticals R&D department for technical assistance and staff of Acculab for providing excellence husbandry service. In particular we would like to thank Alysha Vu and Joe Agnes for proofreading the manuscript. This work was not funded by any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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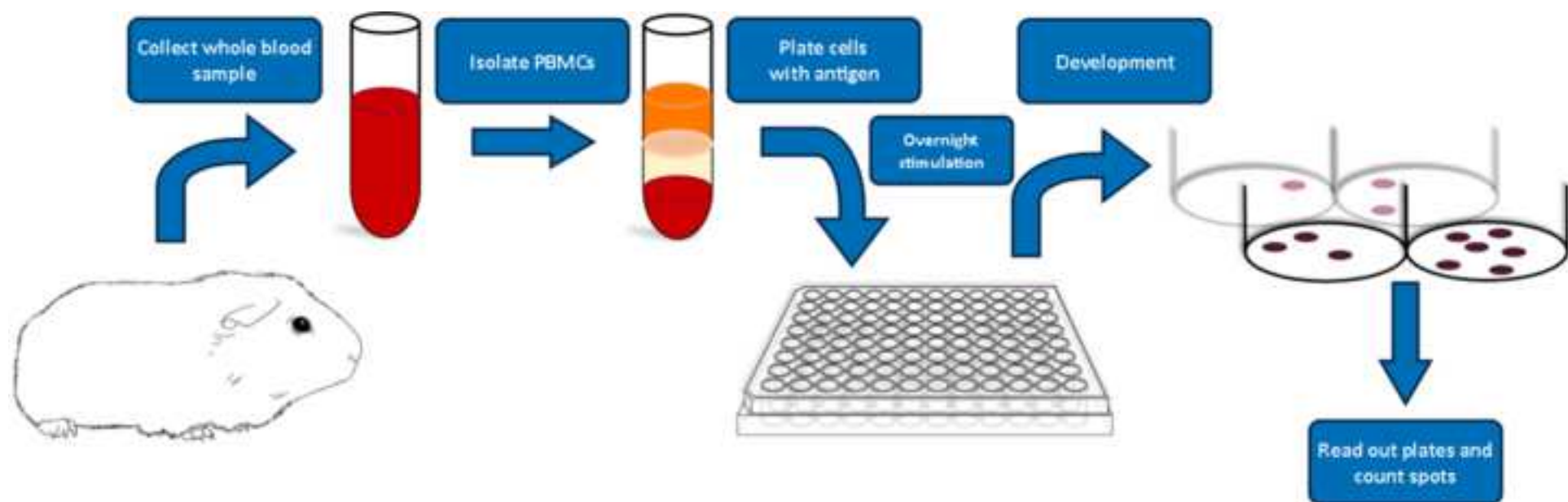
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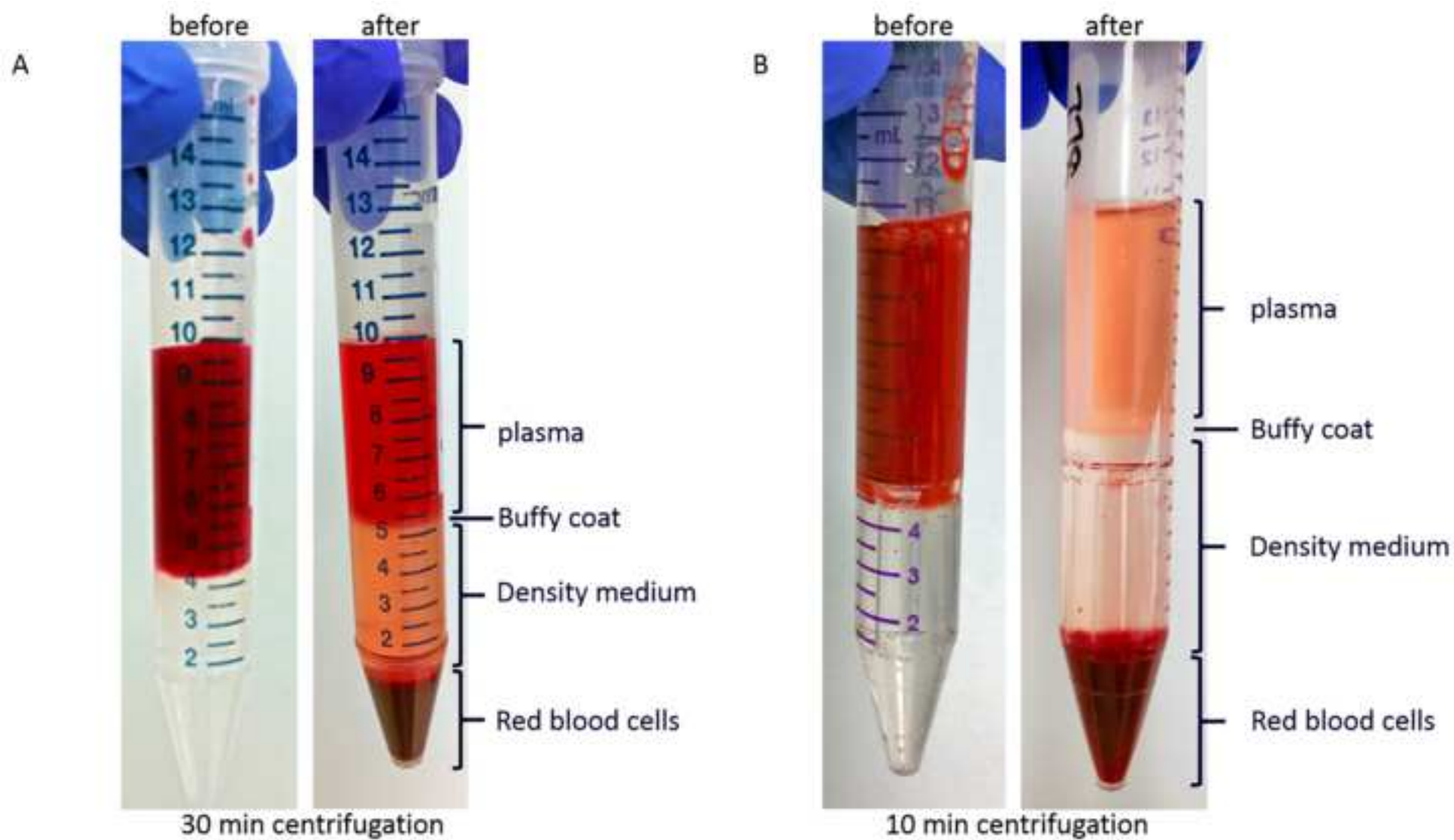
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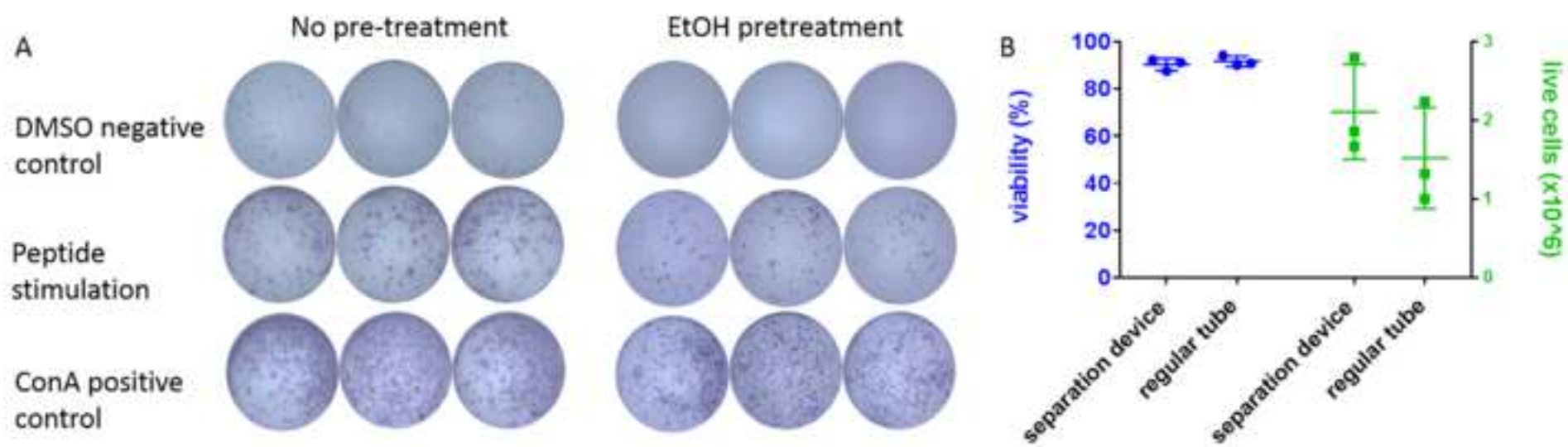
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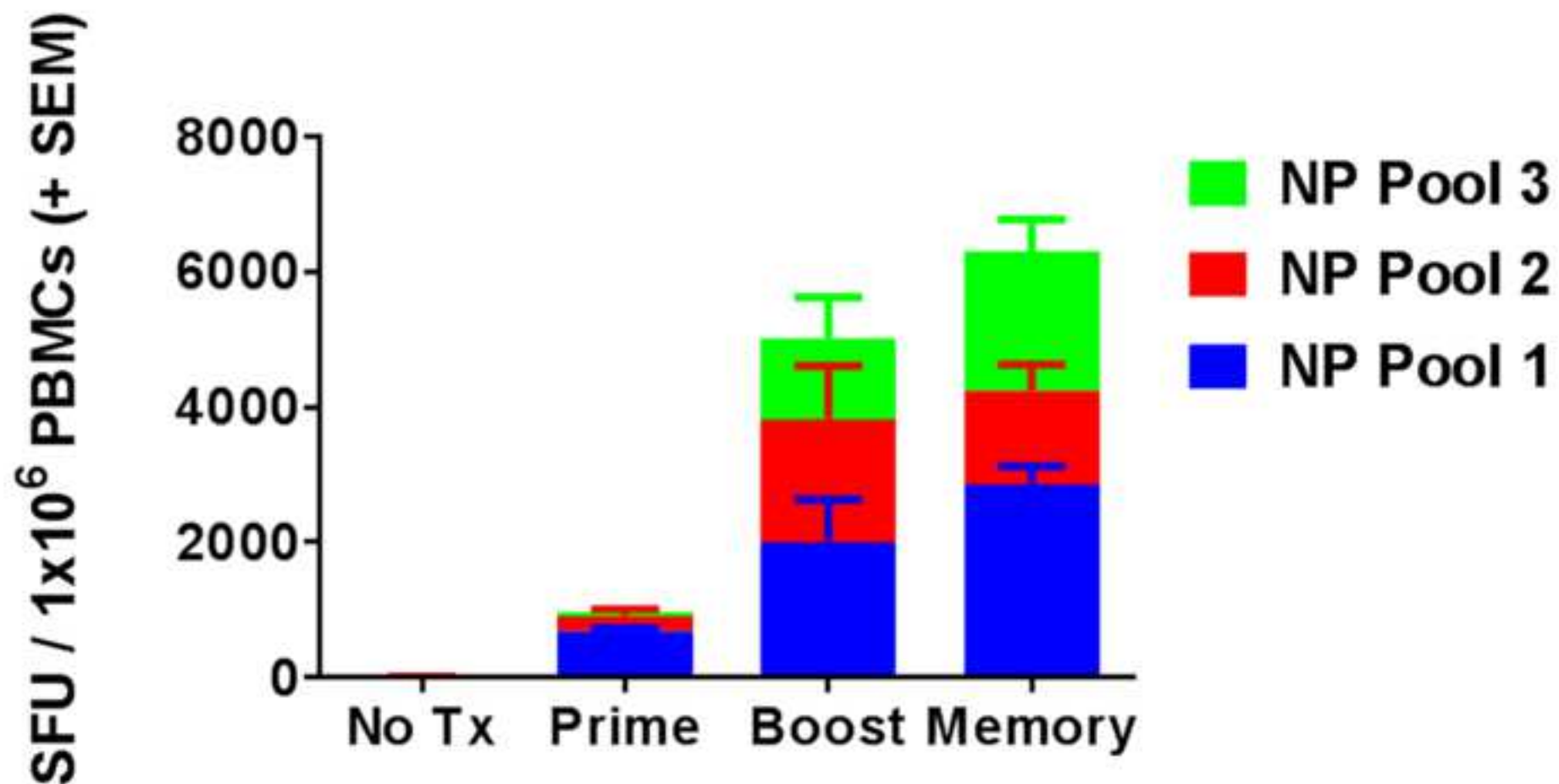
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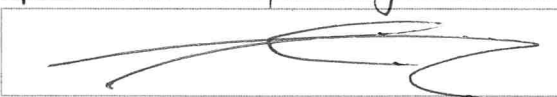
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Dear Dr. Bajaj,

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Author Reply: Email-addresses for each author have been added on page 1 of the revised manuscript.

*4. Please use SI abbreviations for all units: L, mL,  $\mu$ L, h, min, s, etc.*

Author Reply: SI abbreviations are now used throughout the manuscript (lines 160, 182, 183, 203, 209, 210, 223, 226, 230, 233, 235, 239, 246, 250, 255, 259, 266-267, 278, 281, 284, 292, 302, 306, 313, 316, 324, 329, 259, 366, 393)

*5. Please convert centrifuge speeds to centrifugal force (x g) instead of rcf.*

Author Reply: All mentioned centrifuge speeds have been converted from rcf to x g unit (lines 208, 210-211, 228)

*6. Please delete the ^ symbol and use superscript for the exponent (i.e., 6).*

Author Reply: All exponents, including figures, are now in superscript (lines 394, 367, 375, 376,).

*7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: CELLECTRA®, Ficoll-Paque Plus, SepMate™, STEMCELL Technologies, etc.*

Author reply: Throughout the manuscript trademark symbols, brand and company names have been removed (lines 168, 201-206, 208-211, 220, 352, 354-355, 357, 360, 366, 384, 391-394, 399-400, 428, 431-436). Commercial products are referenced in the revised Table for Materials and Reagents.

*8. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please move the discussion about the protocol to the Discussion.*

Author reply: The protocol has been revised to include only action items (lines 143, 158, 163, 194, 253).

*9. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.*

Author reply: More details have been added to the protocol steps (lines 169, 176-177, 185, 188, 199, 201, 203, 215-216, 218, 220-223, 228-231, 255-257, 264, 269, 273, 297-298, 300, 308-311, 318-327, 335-337). In addition, literature references to provide the reader with further details regarding animal anesthesia (lines 148-149, 170-172, 179/180), Mantoux injection technique (lines 164/165), non-terminal bleeding (183) and Trypan-Blue staining (line 237) have been added.

*10. 1.1.1: Please specify the gender and age of guinea pig. Please mention how proper anesthetization is confirmed.*

Author Reply: Guinea pig age and gender have been specified (line 145). We added new sub-step 1.1.2 (line 148/149) to provide instructions detailing anesthesia. We also expanded 1.2.4 (170-172) to specify how to monitor animal's recovery. We provided a reference regarding monitoring of anesthesia in the discussion part (lines 416-420).

*11. 1.2.1: Please describe Mantoux technique or add a reference.*

Author Reply: We have provided a reference describing the Mantoux skin injection technique.

*12. 3.1.3: What happens after the centrifugation? Is supernatant discarded? Please specify throughout.*

Author Reply: The handling of tubes after each centrifugation step in the protocol is detailed, we added new sub-step (3.1.4) to clarify to not pour off any content after density centrifugation.

*13. 3.1.6: What volume of R10 medium is used to wash?*

Author Reply: The volume of R10 medium to use for washes has been specified *in substep 3.2.7 (line 230-231)*.

*14. 3.1.8: Please describe how this is actually done.*

Author Reply: We added a literature reference to provide details about the Trypan-Blue stain (line 237).

*15. 4.1.1, 4.1.2: Please describe how to pre-treat plates with ethanol. What is the incubation temperature?*

Author Reply: The description of ethanol-pretreatment of plates has been provided and incubation temperature specified (lines 246-251).

*16. 4.2.2: This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.*

Author Reply: We modified sub-steps in section 4.2 to clarify what is required for cell plating and to contain only action items (lines 273-288).

*17. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.*

Author Reply: Thank you for the clarification on the use of “notes”. We have converted most of them into protocol steps (lines 185, 196-197, 246-248, 250-251, 314).

*18. Please include single-line spaces between all paragraphs, headings, steps, etc.*

Author Reply: Spacing is now set to ‘single’ for the entire document.

*19. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*

Author Reply: We have highlighted the sections of the protocol that should be video-taped. The video should follow the sequence of steps, e.g. section 4.1.1 through 4.1.7 before section 3.

*20. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*

Author Reply: All relevant details required to perform the step in the highlighting have been provided.

*21. References: Please do not abbreviate journal titles.*

Author Reply: Reference list now displays full journal titles.

*22. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available."*

Author Reply: Table of Equipment and Materials is updated and contains all available information.

#### **Addressing reviewer 1, major concerns**

*"There is one key aspect which undermines the usefulness of publication of this protocol and that is the source of the antibodies. It states that these were generated (without providing the methods) from B cell clones provided by one of the co-authors. For this assay to have any use to the scientific community, there needs to be a commercially available source of the antibody pairs. Without this, each laboratory will need to generate their own reagents in a non-harmonised manner leading to lab-to-lab variation in the assay. Furthermore, there will be labs who would wish to use the assay but cannot because they lack the facilities / resources for antibody production/purification etc. The authors need to comment on this aspect because the assay is completely dependent on the quality of these critical reagents."*

Author Reply: The original clones were developed by Dr Hubert Schaefer, co-author of this manuscript, and his lab. They have addressed the issue of commercialization. Dr Schaefer has an arrangement with the German company ImmunoTools that ensures the availability of the antibodies to the scientific community. Researchers should first place requests to Dr Schaefer's lab, before being referred to ImmunoTools. Production, delivery and invoicing is completely handled by ImmunoTools. The antibodies are available in different formats (biotinylated, HRP-conjugated, fluorochrome-conjugated). Researchers interested in using this assay therefor do not need to have the facilities or resources to produce and purify antibodies. Although a new batch will be manufactured for each placed order, the manufacturing process takes place in a regulated commercial setting that results in consistent product characteristics. We have summarized this procedure in the discussion section (460-463), so readers are informed about how to order the antibodies. Just to clarify: The antibodies used in our lab to generate the data presented in this manuscript were manufactured by GenScript from the same cell-line.

#### **Addressing reviewer 1, minor concerns**

*Line 145: do you mean 'equal' or should it be 'optimal'*

Author Reply: Line 145 (line 208-211 in revised manuscript) has been removed because it was not an action item.

*Line 153: the term 'Trypan Blue exclusion test' would be better here*

Author Reply: Line 153 (line 237 in revised manuscript) We agree with the reviewer and specified the test-name accordingly throughout the manuscript (lines 237, 395, 440).

*Line 187: 'empty' implies removal of all of the contents whereas is only supernatant that is being removed*

Author Reply: Line 187 Changed wording throughout the protocol to clarify, that only supernatant/buffer is removed from wells.

*Lines 190 and 194: it is surely a 0.22um filter, not 22um.*

Author Reply: Lines 190 and 194 (lines 303 and 314 in revised manuscript). The Reviewer is correct, wording changed to clarify the size of filter to be used for this step.

*Lines 200 to 202. Although there is merit in highlighting a particularly toxic reagent, this is not the only chemical in the protocol for which caution should be exercised and there should be a general point about referring to MSDS's and performing risk assessments.*

Author Reply: Lines 200 to 202 (329 to 331 in revised manuscript): We added a general comment regarding MSDSs and PPE at the beginning of the protocol section (line 137-138).

*Line 206: perhaps a recommendation could be given to use a plate reader since this is preferable for storage of the data, more objective analysis etc than manual counting."*

Author Reply: Line 206 (344 of revised manuscript). This is a very valid point and we added a recommendation for an automated system in the discussion part (lines 448-451). We left the decision regarding the counting method in the protocol section more open to the discretion of the protocol-user, since the automated ELISpot plate reader is expensive and not standard lab equipment. The absence of it should not prevent researchers from using the described assay.

#### **Addressing reviewer 2, minor concern**

*"This reviewer believes the authors should state what vaccine was being tested. Some comments suggest it may have been an influenza vaccine. T cell responses differ upon intradermal versus IM delivery of plasmid. A brief discussion of the vaccine being tested is warranted."*

Author Reply: We include a paragraph (473, 482-490, 500) in the discussion section that lists previous work with the same pDNA vaccine used in this manuscript. This literature overview encompasses various species (mice, guinea pig, rabbit) and delivery routes (intra-dermal, mucosal, intra-muscular), in all of them the used pNP vaccine generated robust immune responses in combination with EP. We also made minor additions to introduction (line 69/70) and representative results section (line 391/392) to emphasize that we worked with an Influenza vaccine to generate data for this manuscript.

Again, we thank the reviewers and journal editors for the excellent critique and promptness. We hope we have answered your concerns and applied your suggestions sufficiently to bring this manuscript up to a level ready for video production by JoVE.

Sincerely,

Katherine Schultheis and Trevor Smith

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