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Antigen Presentation Assays to Investigate Uncharacterized Immunoregulatory Genes

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Abstract

Antigen presentation to T lymphocytes is the seminal triggering event of the specific immune response, and poxviruses encode immunomodulatory genes that disrupt this process. Discovery of viral proteins that interfere with steps in the antigen presentation process requires a robust, easily manipulated antigen-presenting and T lymphocyte response system. Use of fresh primary antigen-presenting cells (APC) is preferable because cell lines that can present antigen *in vitro* are often not representative of APC *in vivo* and are typically weak stimulators. To study immunomodulatory poxvirus genes, we have used infected primary rat macrophages to present a model antigen, the myelin basic protein peptide, to a cognate CD4⁺ RsL11 T cell clone. Using this system, viruses can be assessed for difference in immunomodulation, and viral gene functions may also be assayed by comparing effects of wild type virus and mutant viruses (e.g., a deletion in the putative immunomodulatory gene). While antigen presentation can be thought of as a single event, it can also be considered as a larger process comprising multiple steps including: antigen acquisition, antigen processing, peptide loading onto MHC molecules, transport to the surface, MHC binding to T cell receptor, interaction of costimulatory molecules, cell signaling, cytokine synthesis by both cells, and proliferation of antigen specific T lymphocytes. This system allows for the initial determination of whether there is a phenotype and then also allows the stepwise deconstruction of the system to analyze this process at several points to focus in on the mechanism of immunomodulation. We have used this model system to elucidate the function of a highly conserved but previously uncharacterized poxvirus gene that we showed was important for virulence in rodents. The experimental system developed should be broadly applicable to analyzing viral effects on immunity.

Keywords

Antigen presentation; CD4; Immunomodulation; T lymphocyte; Cytokine; Vaccinia; Poxvirus; MHC

1. Introduction

Viruses have an impressive number of strategies for survival, replication, and spread in the host organism. Orthopoxvirus genomes encode approximately 200 genes, many with functions that remain to be elucidated (1). For example, viral virulence genes encode the ability to (1) grow in otherwise restrictive cell types (host range genes) (2–5), (2) block inflammation and immune responses (6–9), (3) inhibit cellular apoptosis (10–12), (4) enhance spread of virus particles using host proteins (13, 14), (5) interfere with cell signaling (15, 16), and (6) globally regulate cellular gene expression (17, 18). Viruses control immune responses to facilitate their survival; including blocking antigen processing and presentation, MHC expression, cytokine and chemokine production, antibody, and cytotoxic T-cell-mediated killing (8, 9).

When a new virulence factor is identified in a virus, it is often desired to determine its effects on immunity. Immune modifiers can be broadly categorized as secreted or cell associated proteins. Secreted factors may also affect neighboring uninfected cells and are likely to fall into the category of growth factors, soluble receptors that bind and inactivate immune components, or soluble factors that bind cell surface receptors, thus delivering an immune-dampening signal or blocking an immunostimulatory signal. Viral proteins that remain cell associated can act intracellularly or intercellularly in a myriad of ways including to inhibit signaling, antigen processing, MHC trafficking, Fas/ligand interactions, and protection from complement-mediated lysis. This chapter describes techniques our laboratory has developed to rapidly assay viral effects on antigen presentation and subsequent macrophage and T lymphocyte activation.

Numerous studies have shown the importance of various aspects of innate and adaptive immunity to defense against viral pathogens. CD4⁺ helper T lymphocytes are important in stimulating and shaping the immune response because they provide “help” to both B and T lymphocyte effector cells. CD4⁺ T cells secrete cytokines that stimulate antibody production and induce immunoglobulin isotype switching in B lymphocytes and also provide help that stimulates the development of CD8⁺ cytotoxic T lymphocytes that kill virally infected cells. While there is evidence that multiple branches of the immune system provide protection from poxvirus infections, the particular importance of CD4⁺ T lymphocytes in survival and recovery from poxvirus infections has been shown in studies using knockout mice, where it was found that the absence of MHC class I molecules or CD8⁺ T cell responses did not diminish protection, but that decreases in CD4⁺ or MHC class II expression caused a loss of protective immunity (19). Therefore, we have explored the effects of poxvirus infection of antigen-presenting cells (APC) and their interactions with CD4⁺ T lymphocytes (8). We expanded this research to include the study of the vaccinia virus A35R virulence gene, which we showed was not required for viral replication in tissue culture but which dramatically decreased virulence in a rodent intranasal challenge model (9, 20).

We wanted to develop an assay that would capture an immune response effect in any of multiple steps in the process of antigen presentation: antigen uptake by the APC, presentation in the context of MHC, macrophage-T cell interaction and mutual stimulation resulting in changes in surface activation proteins, and soluble effector and cytokine

synthesis by both cells. We describe here our use of an antigen presentation cell assay using primary rat APC recruited in vivo to a site of inflammation. These cells are an excellent model, since it is often difficult to culture lines that truly represent APC in vivo. Rats are a good model animal as they are natural hosts of orthopoxvirus infection, they can transmit the viruses to primates (21), their APCs are able to be infected by vaccinia virus (22), and they are large enough to provide sufficient cells for numerous assays. The rat APC are infected with different viruses or virus mutants, pulsed with a model antigen, and then assayed for their ability to stimulate the model antigen specific CD4⁺ T cell line (see Fig. 1) (8, 9, 22). Various cytokines, chemokines, and bioactive mediators may be measured in the supernatants from these antigen presentation assays to determine if there is a decreased response or if the virus or viral gene has altered the character of the immune response. For example, we have used Luminex fluorescent bead technology to measure 23 different cytokines in one 50 μ l aliquot of the harvested supernatants (8, 22). We detected MIP1 α , IL-1 β , GM-CSF, IL-1 α , IL-2, IL-6, IFN- α , IL-17, IL-18, GROK, RANTES, MCP1, and TNF α . Significant quantities of eotaxin, G-CSF, leptin, IL-4, IL-5, IL-9, IL-13, IP-10, and VEGF were not detected. Macrophage and T lymphocyte responses may be easily measured with assays for nitric oxide (NO) and interleukin-2 (IL-2) production, respectively.

We have used this system, or parts thereof, to assess, viral killing of APC, viral replication, induction of apoptosis, and MHC class II and costimulatory protein surface expression (8, 9, 22). This system has the advantage that it is possible to run one assay and determine the end point to see if there is any effect. If there is an effect, the system can then be broken down into steps to determine the location of the block and the changes in the immune response (8, 22).

2. Materials

1. Lewis rats, 3 months to 1 year old.
2. Inactivated *Propionibacterium acnes* (see Note 1).
3. RPMI/10% FBS: RPMI containing 10% FBS, 2 mM glutamine, 100 μ g/ml streptomycin, 100 U/ml penicillin, 50 μ M 2-ME (beta mercaptoethanol) (see Notes 2 and 3).
4. Hanks' balanced salt solution (HBSS).
5. Guinea pig myelin basic whole protein (GPMBP) or GPMBP peptide fragment 68–82 (PQKSQRSQDENPV).
6. CD4⁺ RsL11 T-cell clones (8, 22, 23).
7. 0.1 M sodium nitrite.

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¹*P. acnes* was formerly called *Corynebacterium parvum*. Inactivated *P. acnes* is commercially available as a veterinary product called EqStim (Neogen). This can also be prepared in-house.

²Some lots of FBS are better at supporting the survival and function of immune cells. Unfortunately, no particular supplier of FBS or no known ingredients routinely allow guaranteed immune responses. Therefore, it is best to test serum lots before setting up large experiments. Once you find a FBS lot that works well, save that lot of FBS for your in vitro immunologic experiments.

³Immune cells often require the presence of 5×10^{-5} M beta-mercaptoethanol. We strongly recommend its inclusion in media.

8. Griess reagent: 1% sulfanilamide, 0.1% N-[1-naphthyl] ethylenediamine in 2.5% phosphoric acid (see Note 4).
9. CTLL-2 cells (ATCC # TIB-214).
10. Interleukin 2.
11. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt: 2.0 mg/ml dissolved in PBS (light sensitive) (see Note 5).
12. PMS, phenazine methosulfate: 0.1 mg/ml dissolved in PBS (light sensitive).
13. MTS/PMS: 100 μ l PMS per 2.0 ml of MTS, made fresh prior to addition to the culture plate containing cells.

3. Methods

3.1. Peritoneal Macrophage (APC) Isolation

1. Lewis rats (3 months to 1 year old) are injected intraperitoneally using a 25-gauge needle with 200 μ g of inactivated *P. acnes* diluted in 5 ml of HBSS.
2. Two to three days later, the rats are sacrificed and the peritoneum aseptically opened.
3. Peritoneal exudate cells (APC) are harvested by washing the peritoneal cavity and internal organs three times with 13 ml each time of cold HBSS and aspirating fluids. Use the pipette to gently agitate organs to collect the APC. Be careful not to perforate any blood vessels or organs. You should be able to remove a total of ~40 ml from the peritoneum in a 50-ml conical tube. The cells should be kept on ice from harvest until the time of infection.
4. Pellet the cells by centrifugation at $800 \times g$ for 10 min.
5. Discard media and resuspend the cells in 20 ml RPMI/10% FBS (see Notes 2 and 3).
6. Centrifuge the cells at $800 \times g$ for 10 min.
7. Resuspend the cells in 10 ml RPMI/10% FBS.
8. Count the cells ($2-4 \times 10^7$ cells can be expected per rat).

3.2. Virus Infection of Rat Peritoneal Macrophages

1. Separate required numbers of cells for each experimental group (e.g. wild type, deletion mutants, or uninfected as control) into 15-ml conical tubes (see Notes 6 and 7).

⁴A Griess reagent assay kit is also available from Promega.

⁵MTS is also available as part of a kit from Promega (cell Titer 96).

⁶It is important to use polypropylene conical tubes for infection because the APC will not adhere to them. If tissue culture treated plastics are used for the infection, the APC may adhere and be lost, as they will not be in suspension for addition to the T lymphocytes during the assay.

2. Bring the volume in each tube to approximately 1 ml RPMI/10% FBS. As infection rates are dependent on virus and cell concentration, keep the volumes the same between comparison groups.
3. Calculate the desired multiplicity of infection (MOI, the number of virus per cell, usually 3–10), and add virus (wild type, deletion mutants, or uninfected as control) to the conical tubes containing APC, and mix by flicking the tube. Do not vortex the cells, as they may be damaged. Remember to keep volumes constant in all tubes (see Notes 8 and ⁹).
4. Place conical tubes in 37°C incubator with 5% CO₂. Loosen the tube cap to allow for gas exchange and lay the tube in a nearly horizontal position by resting it on another empty conical tube or pipette to keep liquid from leaking out (see Note 10).
5. During the 2-h incubation with virus, resuspend cells by gentle finger flicking every 20 min, using care to keep the cap in place to maintain sterility. While virus infection should be complete by 2 h, you may wish to leave the infection for longer to allow for adequate protein expression and processing. We have seen good immunoinhibitory activity with 3–5 h infection times (22).

3.3. Loading of APC with Model Antigen

1. After the cells are infected, add 5 ml of warm (37°C) RPMI/10% FBS to the APC in 15-ml conical tubes and pellet the cells by centrifuging at $800 \times g$ for 5 min.
2. Aspirate out media containing unbound virus (see Notes 11 and ¹²).
3. Resuspend cells in 1 ml warm RPMI/10% FBS and add the antigen, guinea pig myelin basic protein or peptide, at a 50 nM final concentration (see Note 13).
4. Incubate for 30 min, with caps loose and tubes placed on the diagonal as described above in a 37°C incubator to allow antigen uptake (see Note 10).
5. Add 5 ml of warm (37°C) media to the APC in 15-ml conical tubes and centrifuge cells at $800 \times g$ for 5 min to wash out antigen (see Note 14).
6. Resuspend 600,000 cells in 600 μ l RPMI/10% FBS to yield 200,000 cells/200 μ l.

⁷We typically do a 1:2 titration of APC from 100,000–1,500 APC per well in triplicate, thus requiring approximately 600,000 cells per group. We recommend to make a titration of APC in order to see a range of responses and maximize the possibility of capturing differences in efficiency of presentation.

⁸The titer of the viruses is very important, as changes in titer may affect the efficiency of antigen presentation. We recommend the use of virus stocks that have been repeatedly titered with high replicate number in order to ensure confidence of titers when comparing two or more viruses. In addition the titer can be reconfirmed on the day of infection.

⁹Purified virus preparations may be employed for infections in order to remove the possibility of measuring effects of trace cytokines or other bioactive molecules carried over in virus preparations from crude cell lysates.

¹⁰The purpose of keeping the tube on its diagonal is to maintain cell viability by allowing for good gas exchange to occur and to make sure that the cells do not settle into a pellet at the bottom of the tube.

¹¹Remember when discarding materials that have come into contact with infectious virus, one needs to autoclave or treat waste with detergent to inactivate virus prior to disposal.

¹²This step is optional, as the RsL11 T cells and CTLL used in later steps are minimally affected by vaccinia virus (22).

¹³You may increase the antigen concentration to increase the stimulation response. We have used from 50 to 500 nM (8), but others have used up to μ M concentrations (24). The GPMBP peptide fragment is less expensive than the whole protein.

¹⁴Washing out the antigen is optional; however, using a defined time of antigen pulse may allow for better detection of changes in efficiency of antigen uptake or processing.

7. Set up 1:2 serial dilutions in triplicate in a flat-bottom 96-well tissue culture plate with 100 μ l of RPMI/10% FBS per well and transferring 100 μ l volume each time (see Fig. 2).
8. Put 200 μ l of the cell suspension in the first well and then dilute by moving 100 μ l from one subsequent well to the next with mixing.
9. Discard the final 100 μ l from the last well so that there will be a volume of 100 μ l in each well after dilutions have been made.
10. Remember to include controls to monitor the proper functioning of the assay. Control wells should include media only, antigen but no APC, and APC and no antigen (see Note 15).
11. Add 100 μ l of RPMI/10% FBS containing 25,000 Lewis rat CD4⁺ RsL11 cells (24) to each well (see Note 16).
12. Incubate the cells for 15–72 h at 37°C in 5% CO₂ to allow for stimulation of both the cells and cytokine secretion.
13. After the desired incubation period, remove the plates from incubator and check media color to note if cells have overgrown (yellow indicates acid production and cell crowding). To take supernatant at a specific time point, tilt plates by propping this figure will be printed in b/w up one edge in a tissue culture hood and carefully and slowly collect 40–50 μ l of supernatants from the top of the deep side of the well (a multichannel pipette works well). Avoid aspiration of cells, since supernatants are desired for analysis of soluble effectors released as the result of antigen presentation (see Notes 17 and ¹⁸).
14. Transfer supernatants from the desired time points directly to an empty 96-well tissue culture plate (or microfuge tubes if needed for another assay format) and assay immediately or freeze for later analysis using the assays described below.
15. Put the plates back in the incubator to incubate for collection of supernatants for later time points. 24 and 48 h are useful time points, but we have detected differences at 12 h and up to 96 h (see Note 19).

3.4. Analysis of Supernatants

Various cytokines, chemokines, and bioactive mediators may be measured as a result of antigen presentation as described in Subheading 1. We describe here two assays for

¹⁵Background in the antigen presentation response will be defined as levels found in the absence of antigen or the absence of either cell type, as all three components are required for antigen presentation responses. The best control is with both cell types and no antigen, but multiple variations missing one component are useful to identify the source of any background.

¹⁶Alternatively, one can add cells in 150 μ l to each well to increase the volume of available supernatants (250 instead of 200 μ l volume), as well as to give the cells more media for longer incubation periods.

¹⁷The volume of fluid collected in each well for each time point should be held constant because the concentration of the biological effectors will be measured following this step. If volumes vary between samples, it will affect concentrations.

¹⁸Check your pipetting technique by looking in the microscope for any evidence of cells in the supernatants you collected. We typically are able to collect a total of 150 μ l (i.e., 50 μ l three times, once for each time point) from wells containing 200 μ l volume without aspirating cells, or a total 200 μ l volume of supernatant from a well containing 250 μ l (if an additional time point will be needed).

¹⁹Take care that at later time points cell crowding and media depletion do not confound interpretation of results. The most strongly stimulated cells will proliferate the fastest and die the fastest creating a plateau effect.

measuring bioactive mediators released as a result of antigen presentation, one for the measurement of IL-2 secreted by T lymphocytes (22, 24), and one for measurement of nitric oxide released by APC after stimulation by the activated T lymphocytes (8, 22).

3.4.1. Measuring the APC Response, Nitric Oxide Measurement—In order to measure the response of the APC to the antigen presentation stimulation from the activated T lymphocytes, supernatants may be assayed for the presence of nitric oxide (22, 25).

1. Supernatants should be in 96 well plates, either fresh or thawed and warmed to room temperature after storage.
2. A solution of 0.1 M sodium nitrite can be used as a positive control and to make a standard curve for sample comparison. To generate a standard curve, make a dilution series from 100 μ M to 0 μ M sodium nitrite on the plate (see Note 20).
3. Add 50 μ l of Griess Reagent to 40–50 μ l of the harvested supernatants (and the wells with the sodium nitrite controls) in a 96-well plate.
4. Wait for 5 min and read the absorbance on a plate reader at 540 nm.
5. NO production should only be detected in supernatants from cultures that contained the model antigen and both cell types. If NO is detected in the absence of any of the three essential components of antigen presentation, there is a contaminant affecting the assay.

3.4.2. Measuring the T Lymphocyte Response, IL-2 Bioassay—The T lymphocyte response to antigen stimulation can be monitored by measuring the IL-2 production. The amount of IL-2 can be easily measured using CTLL cells. CTLL are an IL-2 dependent T cell line that responds to mouse and rat IL-2 by proliferating. Thus, they can be used as an IL-2 bioassay where the absorbance will correlate with CTLL proliferation, IL-2 concentration in the media, and antigen presentation efficiency.

1. CTLL are continuously grown in culture in IL-2 containing media (e.g., RPMI/10%) (see Note 21).
2. For this assay, the cells are used when they are IL-2 deprived and almost dying because this will keep the background low. CTLL should be used when the flask is confluent and it appears that ~50% of the CTLL are dead (see Note 22).
3. You will want 10,000 live CTLL/well, so plan to have at least this many cells. Remove the cells from the flask and place in a 50 ml conical tube.
4. Add the same volume of RPMI (with no IL-2) to the cells and centrifuge at $800 \times g$ for 10 min to wash out residual IL-2.

²⁰Since Griess reagent measures nitrite, sodium nitrite can be used to make a standard curve if you want to quantify how much NO is in the actual samples.

²¹The IL-2 concentrations used to maintain these cell lines will depend on the supplier. We often use an IL-2 in the supernatant from recombinant baculovirus infected cells. The cells are supplied by the ATCC, which recommends “10% T-STIM with Con A, available from Becton Dickinson.”

²²For us, this typically takes 3–4 days of growth in the absence of IL-2. But it depends on how much IL-2 the cells had been growing in prior to putting them in media without IL-2.

5. Aspirate supernatant and resuspend CTLL cells in 10 ml RPMI (with no IL-2) and centrifuge at $800 \times g$ for 10 min.
6. Resuspend CTLL in 5 ml of RPMI (with no IL-2) and count live cells (e.g. by trypan blue exclusion, see Note 23).
7. Dilute the cells to the desired number of cells required for the test samples at a concentration of 10,000 CTLL/well (usually in 150 μ l per well).
8. Add CTLL to 96-well flat-bottom tissue culture treated plate containing supernatants (40–50 μ l) to be tested for IL-2. A multichannel pipette is useful for large numbers of wells or plates.
9. Mix the cells during addition and make sure you add the same number of cells to each well because this will affect your readout.
10. Important control groups are described in Notes 24 and ²⁵.
11. Check the plates using a microscope to ensure that there are approximately the same numbers of CTLL per well.
12. Incubate the plates at 37°C, 5% CO₂ overnight.
13. Check the plates using a microscope. Wait until the cells in the positive control (IL-2 containing wells) appear large and bright and the cells in the negative wells (no IL-2) appear almost dead. Typically, this is about 15–30 h after plating.
14. Add 10 μ l of MTS/PMS to each well. MTS/PMS is light sensitive, so all of the following steps should be done in low light.
15. Once there is a visible color change due to the MTS/PMS addition (usually 6–12 h after adding MTS/PMS), read the absorbance in the plates at 492 nm (and 690 nm reference). We usually read plates twice a day until a color/absorbance plateau begins to be reached.
16. The plates should be observed under the microscope each day for possible contaminations and to ensure that the plate reader and assay are working properly, i.e., wells with the most proliferation have the highest absorbance readings (see Note 26).

3.5. Analysis of Results

If antigen presentation is inhibited by the virus or viral gene, there is expected to be a reduction in the amount of both IL-2 and NO produced, since both of these compounds are

²³For trypan blue staining, pipette 10 μ l of cells into 90 μ l of 0.4% Trypan Blue vital dye (1:10 dilution) and mix. Using a new pipette tip, put 10 μ l of this solution onto a hemocytometer and count bright white cells (dead cells will appear gray or blue). Follow hemocytometer instructions for calculation of cells/ml. Usually (# cells/# squares) $\times$ dilution factor $\times 10^4$ = cells/ml.

²⁴Important negative controls include the following: (a) media only control; three wells containing only 200 μ l RPMI with no IL-2 present and (b) no IL-2 or supernatant control, three wells containing 150 μ l CTLL cells, 50 μ l RPMI (with no IL-2).

²⁵Important positive controls (CTLL and IL-2) include three wells containing 150 μ l CTLL and 50 μ l RPMI CTLL growth media with IL-2 present. An IL-2 standard curve can be constructed using known IL-2 concentrations.

²⁶The best data time point to graph is when the background is low in negative control wells and the positive groups of interest are near their peak absorbance reading but have not yet reached a plateau where the readings stop increasing. The optimal time point for analysis can be conveniently assessed by comparing the sample of interest that gives the highest reading to the negative control. The time point that gives the largest ratio of high reading value–negative control value is probably the best time point to analyze the data.

made as the result of antigen presentation (8, 22). However, the particular effect on the system will give clues as to the mechanism of the viral gene because the APC first stimulates the T lymphocyte and then the activated T lymphocytes activate the APC. For example, if IL-2 production were normal and NO were reduced, it might suggest that the APC were able to present antigen, and the T lymphocytes were able to respond in terms of IL-2, but that either the subsequent response by the APC to T cell mediators was blocked or the T cell secreted a different profile of cytokines after antigen presentation, thus differentially affecting the APC. A number of scenarios are possible, since there is a sequence of events and the virus may block at a particular point.

If an interesting phenotype is discovered, it is next possible to deconstruct this assay system to further elucidate the mechanism of action of the virus or viral gene. The APC can be isolated and assayed in the absence of T lymphocytes by infecting the APC and then stimulating NO production with IFN γ and/or LPS (8). If APC are found to be affected, they can be further assessed for viral effects. We have found that while vaccinia virus does not replicate well in these APC, it does affect metabolism, apoptosis induction, the expression of surface costimulatory markers, MHC, and antigenic peptide, (8, 9, 22, 26). Similarly, the T lymphocyte can be isolated and studied for viral effects by incubating the T lymphocytes with virus and then stimulating the T cells with phorbol myristate acetate, ionomycin, or concanavalin A (8). However, we and others have found that T lymphocytes are not easily affected by vaccinia virus infection (8, 22, 27) and most viral effects may be focused in the APC.

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Fig. 1.

Antigen-presenting cells (APC) are elicited by injection of killed *P. acnes*, and peritoneal exudates cells are harvested 2–3 days later. APC are infected with wild type or mutant virus for 3 h, pulsed with antigen, and added to CD4⁺ T cell clone RsL11. The APC present antigen to the RsL11, and the activated RsL11 produce IL-2 and other cytokines and stimulate the APC to make nitric oxide and cytokines. These bioactive compounds are measured to determine the effects of virus and viral genes on antigen presentation efficiency.

Fig. 2.

Make a 1:2 dilution series by putting 100 µl of media in wells 2–7 of a 96 well plate. We recommend to do this in triplicate to allow for statistical analysis. Add 200 µl of cells to the first well (no media), and take 100 µl from the first well and add it to the second well and mix. Take 100 µl from the second well and add it to the third well and mix. Repeat for the next four wells. When you get to the last well, add the 100 µl, mix, and then remove and discard 100 µl, so that all wells will have a 100 µl final volume. If you begin with 200,000 cells in 200 µl in the first well, you will create a final titration curve with the number of APC per well shown in the figure.