

## Autologous Tumor Cells Engineered to Express Bacterial Antigens

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### Abstract

Cancer immunotherapies are emerging as promising treatment modalities in the management of the disease. As a result, cancer vaccines are considered to be immensely crucial in preventing recurrence, a well-known nemesis in cancer patients because they have the potential to activate memory antitumor immunity. Due to poor antigenicity and self-tolerance, most tumor antigens require interventional vaccine therapies to provide an adequate “danger” signal to the immune system in order to activate a robust, clinically meaningful antitumor immunity. It has been postulated that this requirement may be achieved by providing bacterial and/or viral immunogens to prime this type of immune response. Briefly, we provide here a method of transfecting whole tumor cells with plasmid DNA encoding an immunogenic bacterial protein such as Emm55, which was derived from *Streptococcus pyogenes* (*S. pyogenes*). Subsequent inactivation of the transfected cells by irradiation (100 Gray) prevents replication. This type of whole-cell vaccine, e.g., ImmuneFx™, has demonstrated activity in a murine neuroblastoma model, in canine lymphoma patients with naturally occurring disease, and in many cancer types in companion animals. The protocols described in this chapter provide the necessary materials and methodologies to manufacture such a vaccine.

**Key words** *Streptococcus pyogenes*, Emm55, Autologous, Tumor cells, ImmuneFx™, Canine lymphoma

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## 1 Introduction

Since William Coley’s usage of bacterial extracts in patients with sarcomas to induce antitumor immunity nearly 120 years ago [1], enormous progress has been achieved in tumor biotechnology. The demonstration that immunization with influenza virus-infected tumor cell lysates could induce clinically efficacious antitumor immunity in murine models provided a basis for modern-day autologous/genetically altered tumor cell vaccines [2]. Whole-tumor-cell vaccines have the advantage of being applied to many different types of cancer and can thus be multi-indication cancer vaccines.

The whole-cell vaccine also has the advantage of activating antitumor CD8<sup>+</sup> T cells via direct and indirect (cross-priming) pathways [3]. Unlike other forms of vaccines, i.e., idiotypic, protein, and peptide, whole-tumor-cell vaccines present a plethora of tumor antigens (known and unknown) to the immune system via epitope spreading. Therefore, in theory, whole-cell vaccines should increase clinical efficacy by educating the adaptive immune response to attack tumor cells throughout the body with fewer tumor cells escaping recognition. In support of this contention, an overview of metastatic colon cancer immunotherapy trials demonstrated a higher clinical benefit rate with whole-cell vaccine (46 %) compared to dendritic cell (17 %)-, peptide (13 %)-, or idiotypic antibody-based (3 %) vaccines [4]. In a meta-analysis of tumor vaccines encompassing multiple cancers, Neller and colleagues concluded that whole-cell tumor vaccines provided objective clinical responses in 8.1 % of patients compared to that of 3.6 % with defined antigens [5]. Due to the nature of most tumor antigens, being self-proteins, they are generally not immunogenic and hence not able to mount an efficient antitumor immunity. To augment antitumor immune responses, it is critical, therefore, to introduce a “danger signal” to the tumor cells, which will enable induction of a robust antitumor response. One approach has been to mix whole tumor cells with mycobacteria, bacilli Calmette–Guerin (BCG), e.g., CanVaxin™ for melanoma and Oncovax™ for colon cancer. Another has been to modify whole tumor cells with bacterial genes, e.g., ImmuneFx™. This chapter describes the transfection-mediated modification of autologous tumor cells with genes encoding immunogenic bacterial proteins, such as Emm55, and subsequent inactivation of the transfected cells by irradiation.

Preclinical data from studies in dogs with lymphoma (Table 1) [6] and a variety of tumors in multiple canine and feline breeds (Table 2) demonstrate the clinical usage to date of this multi-indication approach in animals. A Phase 1b clinical study with asymptomatic human patients with indolent non-Hodgkin lymphoma is planned.

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## 2 Materials

### 2.1 Purification of Plasmid DNA

1. EndoFree™ Qiagen plasmid purification kit (Mega kit) with all buffers and accessories along with DNA columns, Buffers P1, P2, P3, FWBR, ER, QBT, QN, QC, TE buffer, Nuclease free water to make 70 % EtOH, RNase and Lyse blue vials (Qiagen, Valencia, CA, USA) (*see Note 1*).
2. Nuclease-free 1.5-mL Eppendorf microfuge tubes.
3. 15- and 50-mL polystyrene tubes.
4. 0.5-, 10-, 20-, 200-, and 1,000-μL pipette tips.

Table 1

The summary of data collected from a canine lymphoma preclinical study using the autologous whole-cell ImmuneFx™ cancer vaccine which uses the *emm55* gene derived from *S. pyogenes*

Canine breed	Age	Sex	Biopsy	Electroporation time (ms)	Electroporation volts (KV)	Transfection (%)	Expression post G418 (%)	Biopsy to vaccination (days)
Labrador	NK	M	Aspirate	0.40	0.348	10-15	>60	ND
Mix breed	10	M	Aspirate	0.40	0.342	8-12	>50	ND
Mix breed	10	M	Aspirate	0.40	0.350	11-14	>70	ND
Retriever	12	M	Aspirate	0.40	0.196	9-12	>55	ND
Shih Tzu	9	F	Aspirate	0.40	0.380	10-14	>65	ND
Doberman	NK	M	Aspirate	0.40	0.380	10-16	>65	ND
Irish Wolf Hound	7	M	Tissue	0.58	0.530	10-16	>65	23
Golden Retriever	6	F	Tissue	0.40	0.352	15-20	60	ND
Braid	5	F	Tissue	0.40	0.348	20	>65	ND
Labrador Mix	8	M	Tissue	0.56	0.520	16	70	ND
Doberman	8	F	Tissue	0.40	0.36	19	65	14
Labrador Retriever	7	M	Tissue	0.58	0.520	20	75	ND
Bulldog	6	M	Aspirate	0.40	0.352	10-14	>75	24
Golden Retriever	12	F	Tissue	0.58	0.530	15-18	>75	16
Dachshund	7	M	Aspirate	0.58	0.530	9-15	>60	15
Vizsla	4	M	Tissue	0.58	0.530	15-18	>50	21
Greyhound	10	F	Tissue	0.60	0.520	15-20	>75	18
Weish Corgi	11	M	Aspirate	0.56	0.520	10-13	>65	33

(continued)

**Table 1**  
(continued)

Canine breed	Age	Sex	Biopsy	Electroporation time (ms)	Electroporation volts (KV)	Electroporation Transfection (%)	Expression post G418 (%)	Biopsy to vaccination (days)
Chow Chow	12	M	Tissue	0.58	0.500	9–13	>55	26
Tibetan Spaniel	9	M	Tissue	0.58	0.530	22	>60	30
Standard Poodle	8	M	Tissue	0.58	0.530	25	>70	61
Labrador Mix	6	M	Tissue	0.60	0.530	27	>65	23
B. Collie Mix	7	M	Tissue	0.56	0.500	19	>75	21
Standard Poodle	9	M	Tissue	0.61	0.520	21	65	18
Aus. Shepherd	6	M	Tissue	0.58	0.520	24	70	18
Airedale	11	M	Tissue	0.60	0.540	27	75	14
Boykin Spaniel	7	M	Tissue	0.41	0.340	10–12	>70	14
English Bulldog	5	M	Tissue	0.45	0.346	13–14	>80	12
Labrador Mix	5	M	Tissue	0.56	0.510	21	>70	14
Boxer	6	M	Tissue	0.46	0.520	17	>70	14
Golden Retriever	7	F	Tissue	14.6	0.076	ND	ND	13
German Shepherd	6	M	Tissue	13.4	0.1	ND	ND	6

The majority of dogs received pcDNA/*emm55* plasmid DNA, while the last two received the pAc/*emm55* plasmid. A variety of breeds, sexes, and ages were treated in this study. The average vaccine regimen consisted of 4-weekly and 4-monthly doses of  $1 \times 10^7$  irradiated cells with a minimum of 10 % transfected cells in a given dose. No patient exhibited any side effects or other illnesses related to this vaccine therapy. An overall improved survival of 222 days over historical (untreated) control of 60 days has been observed in canine lymphoma

**Table 2**  
**Autologous whole-cell ImmuneFx™ cancer vaccine therapy has been utilized in several cancer types in both canine and feline species**

Cancer types	Canine	Feline
Lymphoma	31	1
Squamous cell carcinoma	2	0
Fibrosarcoma	2	3
Transitional cell carcinoma	1	0
Osteosarcoma	1	0
Hemangiopericytoma	3	0
Histiocytic sarcoma	1	0
Cutaneous T-cell lymphoma	1	0
Sebaceous gland carcinoma	1	0
Chondrosarcoma	0	1

5. Isopropanol.
6. 70 % molecular grade ethanol.
7. Molecular grade water.
8. 250-mL conical centrifuge tubes.
9. Luria-Bertani (LB) agar plates and broth with 50 µg/mL kanamycin (*see Note 2*).

### **2.2 Culture Media for Solid Tumors**

1. Dulbecco's minimal essential medium (DMEM) supplemented with the following: 10 % heat-inactivated fetal bovine serum (FBS) (DMEM–10 % FBS), 1× MEM nonessential amino acid solution, 1× penicillin–streptomycin solution, 1 µg/mL hydrocortisone.
2. Wash medium for solid tumors: DMEM medium supplemented with 2× penicillin–streptomycin solution.

### **2.3 Culture Media for Lymphoma Tumors**

1. X-Vivo™ 20 medium supplemented with the following: 10 % heat-inactivated FBS (X-Vivo–10 % FBS).
2. Wash medium for lymphoma or liquid tumors: X-Vivo™ medium supplemented with 2× penicillin–streptomycin.

### **2.4 Tissue Processing of Solid and Lymphoma Tumors**

1. Sterile scissors and forceps.
2. 50-mL conical tubes.
3. 15-mL conical tubes.
4. 70 % ethanol.

5. Collagenase/hyaluronidase.
6. T75 tissue culture flasks.
7. Sterile distilled water.
8. DNase I.
9. 0.4 % Trypan blue.
10. Sterile 100×20-mm tissue culture Petri dishes.
11. 2-mL cryogenic vials.
12. Recovery cell culture freezing medium (Invitrogen, Life Technologies, Carlsbad, CA, USA).
13. 5-, 10-, 25-mL sterile pipettes.
14. 0.22- and 0.45- $\mu$ m syringe filters.
15. 12-mL syringes and sterile 20 mL luer-lock syringes.
16. 0.25 % Trypsin–EDTA solution. Bring to room temperature in the hood prior to use.
17. Hanks' Balanced Salt Solution (HBSS).
18. RBC Lysis buffer.
19. Fico/Lite-LymphoH.
20. Sterile 18 G×1.5 in. needles.
21. Sterile 100×20 mm tissue culture Petri dishes.
22. T175 tissue culture flasks.
23. 2-mL cryogenic vials.

### **2.5 Transfection of Plasmid DNA into Tumor Cells**

1. Purified plasmid DNA.
2. 50-mL conical tubes.
3. Mirus electroporation buffer.
4. Recovery cell culture freezing medium.
5. 1.5-mL Eppendorf tubes.
6. Gene Pulser 0.4-cm cuvettes.
7. 0.4 % Trypan blue.
8. 2-mL cryogenic vials.
9. 10- and 200- $\mu$ L pipettes.
10. 10- and 200- $\mu$ L barrier pipette tips.
11. T175 tissue culture flasks.
12. CellRad irradiation device (Faxitron, Tucson, AZ, USA).

### **2.6 Immunofluorescence Assay for Transfected Cells**

1. Dulbecco's PBS (DPBS).
2. Heat-inactivated FBS.
3. 4 % formaldehyde: 1 mL of formaldehyde in 8 mL of DPBS (can be stored at room temperature in the dark).

4. Staining buffer: 2.5 mL of FBS in 47.5 mL of DPBS (5 % FBS) and 50 mg of sodium azide (0.1 %).
5. Permeabilization buffer: 10 mg of saponin in 10 mL of staining buffer.
6. FITC-conjugated mouse IgG isotype control.
7. FITC-conjugated anti-CD45 monoclonal antibodies (*see Note 3*).
8. Monoclonal antibodies against the bacterial protein of interest.
9. 5-mL tubes.
10. 200- $\mu$ L pipette tips.
11. 5-mL pipettes.
12. Prolong<sup>®</sup> Gold anti-fade reagent with DAPI.
13. Cytocentrifuge Cytospin 3.

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### 3 Methods

#### 3.1 Extraction of Endotoxin-Free Plasmid DNA

1. The following steps are to be carried out after overnight culture of plasmid DNA-transformed *Escherichia coli* in LB broth at 37 °C in a shaker incubator. Just before Mega kit plasmid extraction, as described by the kit manufacturer, follow these steps:
  - (a) Add RNase A solution to Buffer P1.
  - (b) Check Buffer P2 for any SDS precipitation.
  - (c) Pre-chill Buffer P3 to 4 °C.
  - (d) Optional: Add LyseBlue reagent to Buffer P1.
2. Collect 500 mL of bacterial culture, and pellet the bacteria in 250-mL conical tubes by centrifuging at 6,000  $\times g$  for 15 min at 4 °C. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained. Dispose of the supernatant after autoclaving. Resuspend the bacterial pellet in 50 mL of Buffer P1.
3. Add 50 mL of Buffer P2, mix thoroughly by inverting 6 $\times$  and incubate for 5 min at room temperature.
4. During the incubation time, screw the QIAfilter Cartridge onto a glass bottle with a 45-mm neck and connect it to a vacuum source.
5. Add 50 mL of pre-chilled Buffer P3, and mix thoroughly by inverting 6 $\times$ .
6. Pour the lysate into the QIAfilter Cartridge, and incubate for 10 min. Pull the liquid through by applying vacuum. Add 50 mL of Buffer FWB2 to the QIAfilter Cartridge, and gently stir the precipitate using a sterile spatula or a 5-mL pipette. Apply vacuum again until all the liquid has completely passed through.

7. Add 12.5 mL of Buffer ER to the filtered lysate, mix by inverting the bottle approximately 10× and incubate on ice for 30 min. Equilibrate a QIAGEN-tip 2500 by applying 35 mL of Buffer QBT, and allow the column to empty by gravity flow.
8. Apply the filtered lysate from **step 7** to the QIAGEN-tip, and allow it to enter the tip. Wash the QIAGEN-tip with 200 mL of Buffer QC.
9. Elute the bound DNA with 35 mL of Buffer QN.
10. Precipitate the DNA by adding 24.5 mL (0.7 volume) of room-temperature isopropanol to the eluted DNA. After mixing thoroughly, centrifuge at 6,000×*g* for 60 min at 4 °C to pellet the DNA.
11. Wash the DNA pellet with 7 mL of endotoxin-free 70 % ethanol (kept at room temperature) and centrifuge at 6,000×*g* for 60 min at 4 °C. Carefully decant the supernatant without disturbing the pellet.
12. Air-dry the pellet in a sterile hood for 10–20 min, and dissolve the plasmid DNA in either endotoxin-free TE Buffer or water.
13. Prior to use in transfecting patient tumor cells, each lot of plasmid must be certified according to established criteria. The following are the specifications we use:
  - (a) Quantification of plasmid (1 mg/mL).
  - (b) Gel electrophoresis (>50 % supercoiled DNA).
  - (c) Restriction enzyme digestion and agarose gel (to document the presence of the gene insert).
  - (d) Endotoxin (<100 EU/mg plasmid DNA).
  - (e) Sterility test (should be negative).
  - (f) Sequencing (must match the reference sequence).

### **3.2 Processing Solid Tumors**

1. Wear protective clothing—lab coat, gloves, and face mask.
2. Most of the procedure should be performed under a laminar flow hood.
3. Spray specimen container with 70 % ethanol, and place solid tumor tissue sample under the hood.
4. Place sterile scissors and forceps in individual 50-mL tubes containing 40 mL of 70 % ethanol, and let tubes stand upright in a 50-mL tube rack.
5. Drain forceps by holding them vertically over a clean paper towel. Avoid touching the paper towel.
6. Use forceps to collect tumor from specimen container, and place it in a 100×20-mm Petri dish with 5–10 mL of wash medium. Replace forceps in ethanol.
7. Using sterile scissors, mince the tissue into approximately 1.0 mm<sup>3</sup> pieces.



8. Place all minced tissue pieces in a 50-mL conical tube(s), secure cap, and invert 3–5 times.
9. Pulse centrifuge at  $680\times g$ . Once the speed reaches  $680\times g$ , stop the centrifugation. Repeat this process 3–5 $\times$  until the medium appears clear.
10. Prepare 10 mL of culture medium containing collagenase/hyaluronidase (2–3 $\times$  concentration depending on the texture of the tissue) and 50  $\mu\text{g}/\text{mL}$  of DNase I (*see Note 4*). Filter solution through the 0.45- $\mu\text{m}$  syringe filter and then through a 0.22- $\mu\text{m}$  syringe filter into a sterile 15-mL conical tube.
11. Discard the supernatant, add the filtered collagenase/hyaluronidase solution to the pellet, and place the tube into a 37 °C water bath.
12. Gently swirl the tube every 15–30 min for cell release, and determine viability. A total of 2–4 h may be needed to digest 5 mL of minced tissue pellet, depending on the texture and type of tissue. It is difficult to digest the entire tissue. Some will remain as undigested pieces. Carefully collect the collagenase/hyaluronidase solution containing single cells, leaving the undigested tissue sections undisturbed. Continue digesting partially digested pieces to attain a sufficient number of cells. If necessary, use fresh enzymes (*see Note 4*).
13. Place single-cell suspension in a 50-mL conical tube and add 30–40 mL of wash medium. Also, add 30–40 mL of wash medium to the undigested tissue sections.
14. Centrifuge both tubes at  $680\times g$  for 8 min.
15. Repeat washing the “single”-cell pellet and undigested tissue pieces two additional times.
16. Determine cell viability and total cell number.
17. Resuspend the single-cell pellet and undigested tissue pieces in 10 mL of DMEM–10 % FBS culture medium each.
18. Seed the cells in 1 or multiple T75 tissue culture flasks at a concentration of  $1\text{--}10\times 10^5$  cells per flask in 15–25 mL of medium.
19. Incubate the flasks at 37 °C in humidified atmosphere of 5 %  $\text{CO}_2$ .
20. Monitor cultures daily for cell growth. Solid tumor cells adhere to the flasks.
21. When the tumor cells are 80 % confluent, prepare for splitting the culture by trypsinization.
22. Expand the cells to reach the desired cell number. Every solid tumor type varies in expansion capabilities (*see Note 5*).
23. When sufficient cells are attained, proceed to transfection.

### **3.3 Trypsinization of Adherent Cells**

1. Under a laminar flow hood, remove the spent medium, and dispose of it in 10 % bleach. Add 15 mL of HBSS to gently wash the flask, and discard the medium.
2. Add 5 mL of 1× trypsin to each T75 flask (*see Note 6*).
3. Place the flasks at 37 °C for 5–10 min.
4. Remove the flasks and shake for 30 s with gentle tapping with the palm of hand.
5. Observe under a microscope to ascertain that cells are in suspension.
6. Add 10 mL of sterile DMEM–10 % FBS to the flask and mix gently by using a 10-mL pipette.
7. Transfer the cells to a 50-mL conical tube, add an additional 15 mL of DMEM with 10 % FBS, and centrifuge at  $680 \times g$  for 10 min. Discard the supernatant.
8. Wash the cell pellet at least two more times with 10 mL of DMEM–10 % FBS.
9. Determine cell viability, and continue cell expansion until the required cell number is achieved.

### **3.4 Processing Lymphoma Tumor Specimens**

1. Wear protective clothing—lab coat, gloves, and face mask. And perform the procedures under a laminar flow hood.
2. Spray the specimen container with 70 % ethanol and place under the hood.
3. Place sterile scissors and forceps in individual 50-mL tubes containing 40 mL of 70 % ethanol. Let tubes stand upright in a 50-mL tube rack.
4. Drain forceps by holding them vertically over a paper towel. Avoid touching the paper towel.
5. Gently collect the lymph node (LN) from the container and place in a 100×20-mm Petri dish. Replace the forceps in the ethanol.
6. Attach an 18 G luer-lock needle to a 20-mL syringe.
7. Using a 25-mL sterile pipette, add 20 mL of X-Vivo™ wash medium to a Petri dish with the LN.
8. Aspirate the medium into the 20-mL syringe. Holding the LN with the forceps, make several holes in the LN by gently inserting the needle through the LN capsule and inject medium into the LN. The suspended LN cells will exit through the holes.
9. Repeat this aspiration/injection process using X-Vivo™ wash medium until most of the cells have been released. Collect the cell-containing medium in fresh 50-mL conical tubes.
10. Centrifuge the cells at  $680 \times g$  for 8 min.

11. If applicable, combine all cell pellets into one 50-mL tube and centrifuge at  $680 \times g$  for 8 min.
  - (a) Discard the supernatant. Examine cell pellet, and determine the degree of contamination with RBC. If significant RBC are present, remove using RBC lysis buffer. Depending on the size of pellet, add 5–10 mL lysis buffer to the cell pellet mixing gently with a pipette for 3–5 min. Add 20–30 mL X-Vivo™ wash medium to the cell suspension. Centrifuge the cell suspension at  $680 \times g$  for 8 min. Discard the supernatant. The cell pellet should appear white.
  - (b) Resuspend cell pellets in X-Vivo™ wash medium, and determine cell density and viability. If viability is poor (<60 %), use a one-step Ficoll gradient to enrich viable cells. This step also removes RBC. Depending on cell concentration, resuspend the LN cells in 10 mL aliquots. Using a 10-mL pipette carefully float the cell suspension onto 10 mL of Ficoll. Centrifuge the Ficoll gradient at  $1,890 \times g$  for 25 min at 20 °C. Locate the band of cells at the interface of the X-Vivo™ culture medium and Ficoll. Using a 5-mL pipette, carefully remove these cells and place in a clean 50-mL tube. The majority of dead cells will form a pellet at the bottom of the Ficoll layer along with RBC. Add 20 mL of X-Vivo™ wash medium to the viable cells, and centrifuge at  $680 \times g$  for 8 min. Discard the supernatant, resuspend the pellet in 20 mL of X-Vivo™ wash medium, and repeat cell viability count.
12. Seed T175 tissue culture flasks with a lymphoma cell concentration of  $10\text{--}20 \times 10^7$  cells per flask. Add fresh X-Vivo™ medium with 10 % FBS to give a total volume of 45 mL/flask.
13. If sufficient cells are available, proceed to transfection.

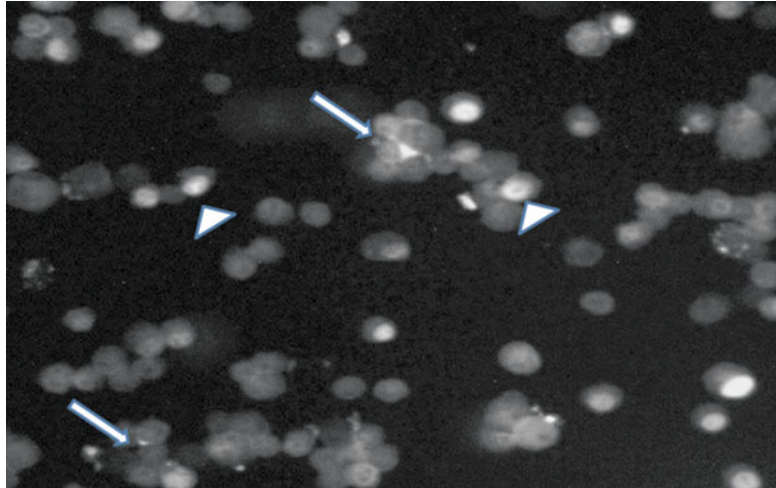
### **3.5 Transfection of Plasmid DNA into Tumor Cells**

1. Using trypan blue, determine the viable number of tumor cells.
2. Reconstitute the cells in a sterile 50-mL conical tube to a final cell concentration  $10\text{--}20 \times 10^6$  cells/300  $\mu$ L of Mirus buffer for lymphoma cells or  $5 \times 10^6$  cells/300  $\mu$ L of Mirus buffer for solid tumor cells.
3. Add 5–20  $\mu$ g of plasmid DNA per 300  $\mu$ L of Mirus buffer reaction (*see Note 7*).
4. Mix the cell/plasmid mixture using a barrier pipette tip, and transfer the mixture to a 0.4-cm sterile gene pulsing cuvette in Mirus buffer.
5. Pulse samples at 240 V and 750  $\mu$ F (*see Note 7*). This will vary for different cell types and may depend on the equipment. A balance has to be reached between transfection efficiency and cell death.

6. Transfer the cells from each cuvette into T175 flasks containing 30–40 mL of culture medium. For small cell concentrations,  $\leq 1 \times 10^6$ , use T25 flasks.
7. Place the flasks in a 37 °C incubator in a humidified atmosphere of 5 % CO<sub>2</sub>.
8. Allow cells to express the protein of interest by culturing for 48 h.
9. After 48 h, collect 10–15  $\times 10^6$  cells/mL for  $\gamma$ -irradiation (100 Gray) (*see Note 8*).
10. Following irradiation, wash the cells with culture medium, adjust the cell concentration, and aliquot in cryogenic vials at cell concentrations appropriate for vaccine applications, e.g.,  $1 \times 10^7$ /mL with recovery cell culture freezing medium.
11. Store at –80 °C for 24 h in a freezing container (*see Note 9*), and then transfer to a liquid nitrogen dewar for long-term storage of transfected tumor cells in the vapor phase of liquid nitrogen.

### **3.6 Immunofluorescence Assay for Transfected Cells**

1. Resuspend cells in 5-mL tubes in 3 mL of staining buffer and centrifuge at  $680 \times g$  for 5 min.
2. Discard the supernatant. Resuspend the cell pellet in 100  $\mu$ L of 4 % formaldehyde to fix the cells.
3. Incubate at 4 °C for 20 min in the dark.
4. Wash the cells 2 $\times$  in 3 mL of staining buffer.
5. Resuspend the pellet in 100  $\mu$ L of staining buffer. Add 10  $\mu$ L of isotype antibody to one tube and a combination of 5  $\mu$ L of anti-“protein of interest antibody” to another tube. Incubate for 30 min at 4 °C in the dark.
6. Wash the cells 2 $\times$  in 3 mL of staining buffer.
7. Resuspend the cells, isotype, and protein of interest tubes in 100  $\mu$ L of permeabilization buffer. Add the combination of 5  $\mu$ L of anti-protein of interest antibodies. Incubate for 30 min at 4 °C in the dark.
8. Wash the cells 2 $\times$  in 3 mL of staining buffer.
9. Resuspend the cells in 500  $\mu$ L of staining buffer.
10. Prepare slide-absorbent filter pad-adaptor assembly for cytocentrifugation.
11. Add 100  $\mu$ L of cells into each slide assembly.
12. Spin the slides at  $300 \times g$  for 3 min.
13. Once cytocentrifugation is complete, allow the slides to dry for 5 min.
14. Over the cell spot on the slide, add 25  $\mu$ L of anti-fade reagent, and gently place a cover slip over the anti-fade reagent (Fig. 1).



**Fig. 1** A representative immunofluorescence of T lymphoma cells transfected with pAc/emm55 plasmid. Expression of Emm55 protein is identified using a combination of two monoclonal antibodies directed against Emm55. Both cytoplasmic (*arrow head*) and surface expression (*arrows*) can be seen

15. Observe the fluorescence using an FITC filter.
16. Save images of cells, both under phase contrast regular lighting and with FITC filter, for each field. Have at least 200 cells from different fields of view for estimating efficacy.
17. Once images are saved, count the total number of cells under phase contrast regular lighting and the number of cells exhibiting fluorescence under FITC.
18. The transfection efficiency is calculated as follows:

$$\frac{\text{Number of cells fluorescing}}{\text{Total number of cells in the same field}} \times 100$$

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## 4 Notes

1. Endotoxin-free plasmid extraction is crucial in therapies intended for clinical trials. Although autologous whole-cell ImmuneFx™ cancer vaccine therapy does not involve direct plasmid DNA injection into patients, our plasmid specification accepts only <100 EU/mg of plasmid.
2. The usage of ampicillin may not be acceptable in clinical trials. Therefore, it is recommended that penicillin and other beta-lactam antibiotics be avoided during production due to the risk of serious hypersensitivity reactions in patients and conferring unnecessary risk of antibiotic resistance to environmental microbes [7].

3. Mouse anti-canine CD45 antibody, clone CD12.10C12, recognizes the canine homolog of CD45, a glycoprotein of approximately 200 kDa which is expressed by cells of hemato-poietic origin. This antibody serves as a positive control for lymphoma cells in our immunofluorescence assay.
4. The DNase is added to prevent clumping of the cells, and it also helps to reduce viscosity resulting from DNA released from damaged cells. The collagenase/hyaluronidase enzyme mixture is supplied as a 10× solution, and it is optimized for overnight digestion of mouse mammary tissue by the manufacturer. We have used the enzyme mixture in at least eight different types of canine solid tumors (Table 2). Depending on tumor texture, 2–3× concentrations have been used and digestion duration varied from 2 to 4 h. In some instances, the partially digested pieces of tumor tissue required a second helping of fresh enzymes. Due to our processing requirements, no overnight digestion is carried out.
5. The rate of growth of solid tumor cells *in vitro* is substantially different among tumor types and among the tumor masses of the same tumor type in different patients. While chondrosarcoma, hemangiopericytoma, and fibrosarcoma cells exhibit fast growth, all epithelial tumors we have processed have provided few cells following digestion and slower growth rates in cultures.
6. While fibroblastic sarcoma, hemangiopericytoma, and chondrosarcoma cells lift off the plate with 1× trypsin, most epithelial cells require 2–3× trypsin to detach from the plastic surface. This property of epithelial cells has been utilized in eliminating fibroblast cells from epithelial cell cultures, i.e., differential trypsinization.
7. The electroporation parameters described here are for BioRad Genepulser II. Depending on the electroporation equipment used, the parameters have to be optimized. Besides the equipment, the optimization protocols should consider factors such as cell type, cell number, plasmid dose per reaction, electroporation buffer, and the percent transfection desired. A balance has to be achieved between attaining a desirable transfection level and the resulting cell death due to the parameters set.
8. A cellular vaccine such as ImmuneFx™ must be inactivated prior to administration into patients to avoid replication of tumor cells. In order to determine the optimal radiation dose with CellRad (an X-ray-based irradiation device), a range of doses (50, 100, and 150 Gray) with primary tumor cells and a lymphoma cell line were studied and a dose of 100 Gray was found to be optimal by AlamarBlue assay.
9. It is critical that the cell cryo-storage protocol include a freezing container with isopropyl alcohol (maintained at room tem-

perature when not in use) as it enables a decrease of  $-1^{\circ}\text{C}$  per minute in a  $-80^{\circ}\text{C}$  freezer. This prevents ice crystal formation within the cells and thereby protects cells from a loss of integrity and reduction in viability.

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