Video Article

Development of a Colloidal Gold-based Immunochromatographic Test Strip for Detection of Cetacean Myoglobin

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Abstract

This protocol describes the development of a colloidal gold immunochromatographic test strip based on the sandwich format that can be used to differentiate the myoglobin (Mb) of cetaceans from that of seals and other animals. The strip provides rapid and on-the-spot screening for cetacean meat, thereby restraining its illegal trade and consumption. Two monoclonal antibodies (mAbs) with reactivity toward the Mb of cetaceans were developed. The amino acid sequences of Mb antigenic reactive regions from various animals were analyzed in order to design two synthetic peptides (a general peptide and a specific peptide) and thereafter raise the mAbs (subclass IgG_1). The mAbs were selected from hybridomas screened by indirect ELISA, western blot and dot blot. CGF5H9 was specific to the Mbs of rabbits, dogs, pigs, cows, goats, and cetaceans while it showed weak to no affinity to the Mbs of chickens, tuna and seals. CSF1H13 can bind seals and cetaceans with strong affinity but showed no affinity to other animals. Cetacean samples from four families (Balaenopteridae, Delphinidae, Phocoenidae and Kogiidae) were used in this study, and the results indicated that these two mAbs have broad binding ability to Mbs from different cetaceans. These mAbs were applied on a sandwich-type colloidal gold immunochromatographic test strip. CGF5H9, which recognizes many species, was colloid gold-labeled and used as the detection antibody. CSF1H13, which was coated on the test zone, detected the presence of cetacean and seal Mbs. Muscle samples from tuna, chicken, seal, five species of terrestrial mammals and 15 species of cetaceans were tested in triplicate. All cetacean samples showed positive results and all the other samples showed negative results.

Video Link

The video component of this article can be found at https://www.jove.com/video/53433/

Introduction

Historically, cetacean meat has been consumed in many parts of the world and this consumption continues today[1]. Due to the trophic level of cetaceans, high levels of mercury and other toxins are known to be present in their meat[2]. Therefore, the consumption of cetacean meat could lead to a health problem not only for high-risk groups such as pregnant women but also for the general population[3]. Furthermore, the contamination of cetacean meat with zoonotic or potentially zoonotic pathogens can also occur during its processing and storage[4]. It is difficult even for experienced agents to identify cetacean meats by their appearance alone. Therefore, a reliable scientific method of identification is required to differentiate cetacean meat from other meats. This would help to limit the consumption of cetacean meat.

Current methods of species identification include molecular techniques and immunological methods. Molecular techniques, such as polymerase chain reaction (PCR) and DNA sequencing, can be used to identify samples not only from raw meat[5] and decomposed samples[6] but also from processed foods such as cooked sausage and feedstuffs[7,8]. Immunological methods, such as enzyme-linked immunosorbent assay (ELISA), are commonly applied in food production to detect the meat content of, for example, pork[9], beef[10] and catfish[11]. PCR-based DNA analysis for the identification of cetacean meat is available[12], and has helped prevent the illegal international trade of cetacean meat in Japan, South Korea, the Philippines, Taiwan, Hong Kong, Russia, Norway, and the United States[1]. These methods are effective and reliable, but they can take hours or days to complete and involve laborious steps. The identification of cetacean meats is usually based on molecular techniques and there is currently no immunological method available. For regulatory agencies, it is highly desirable to develop a dependable and rapid technique that can be used in the field to identify cetacean meats.

Immunochromatographic strips are used as detection tools with the advantage of producing rapid result via a simple protocol that is suitable for use in the field. The principles of the immunochromatographic strip and ELISA are very similar, and includes antibodies, antigens and labels. Many different labels such as colloidal gold, carbon and latex have been used in the development of immunochromatographic strips. At present, this method is commonly used for detecting antibiotics, toxin, bacteria and viruses[13], but it is rarely used for identifying proteins in meat[14,15]. Here we propose a lateral-flow chromatographic enzyme immunoassay for rapid detection of cetacean myoglobin (Mb).

Protocol

Ethics Statement: The study was performed in accordance with international guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of National Chiayi University, approval ID: 99022. The cetacean sample use was permitted by Council of Agriculture of Taiwan (Research Permit 100M-02.1-C-99).

1. Muscle Sample Preparation and SDS-PAGE

Note: Muscle samples from 23 species including 16 species of marine mammals, 5 species of terrestrial mammals, tuna and chicken were used in this study (**Table 1**). The cetacean muscle samples were obtained from stranded individuals, fishery bycatch, and confiscation. Rabbit, rat, dog, and chicken muscle tissues were obtained from Animal Disease Diagnostic Center of National Chiayi University. Samples of beef, pork, lamb, and tuna were purchased from a local supermarket. The muscle sample of harbor seal (*Phoca vitulina*) was provided by Farglory Ocean Park. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate soluble proteins with different molecular weights in muscle samples.

1. Store all samples at -20 °C until use.
2. Pre-cool mortar at -20 °C. Then put 3 g of frozen muscle sample into it.
3. Homogenize the sample with 10 ml of cool phosphate-buffered saline (PBS) using a tissue homogenizer.
4. Centrifuge the homogenized sample at 10,000 x g for 10 min at 4 °C. Collect the supernatants and store it at -20 °C until use.
5. Prepare 5 ml of 5% stacking gel (3.07 ml of distilled water, 1.25 ml of 4x upper gel buffer of pH 6.8, 625 µl of 40% acrylamide, 50 µl of 10% ammonium persulfate (APS), and 5 µl of tetramethylethylenediamine (TEMED)) and 10 ml of 15 % separating gel (3.65 ml of distilled water, 2.5 ml of 4x lower gel buffer of pH 8.8, 3.75 ml of 40% acrylamide, 100 µl of 10% APS, and 4 µl of TEMED).
    1. In the electrophoresis cell, pour stacking gel (5% acrylamide) on top of the separating gel (15% acrylamide) after the latter has solidified. Insert a gel comb in the stacking gel.
6. Perform PAGE according to the following conditions: initial run condition: 100 volts, 20 min and final condition: 120 volts, 40 min.
7. Stain the gel with Coomassie brilliant blue at room temperature for 30 min until the gel is uniformly blue in color.
   Note: Staining is complete when the gel is no longer visible in the dye solution.
8. Destain it with acetic acid solution (10%) at room temperature for 1 hr. Bands will begin to appear. Continue destaining at 4 °C overnight until background is clear.

2. Peptide Synthesis and Monoclonal Antibody Production

1. Retrieve the amino acid sequences of Mb from GenBank including tuna, chicken, ostrich, domestic mammals, seal and 18 species of cetaceans (**Table 2**).
2. Align the sequences using proper software[16]:
    1. Launch the Alignment Explorer by selecting the *Align: Edit/Build Alignment* on the launch bar; select *Create New Alignment* and click *OK*. A dialog will appear asking "Are you building a DNA or Protein sequence alignment?"
    2. Click the button labeled "*Protein*"; select *Data: Open: Retrieve sequences from File* and select sequence file; select the *Edit: Select All* menu command to select all sites for every sequence in the data set for creating a multiple sequence alignment.
    3. Select *Alignment: Align by ClustalW* from the main menu to align the selected sequences data using the ClustalW algorithm; select "BLOSUM" as the Protein Weight Matrix then click the *OK* button.
3. Analyze the sequence alignment:
    1. Focus on 5 antigenic reactive sites[17]: site 1 (AKVEADVA, 15-22), site 2 (KASEDLK, 56-62), site 3 (ATKHKI, 94-99), site 4 (HVLHSRH, 113-119) and site 5 (KYKELGY, 145-151) and find the fragment conserved among cetaceans. An * (asterisk; consensus symbol in the alignment (Protocol 2.2.3)) indicates positions which have a single, fully conserved residue. The following conserved fragments in cetaceans were found: sequence KASEDLKKHG (which includes site 2) and sequence HVLHSRHP (which includes site 4).
4. Synthesize candidate sequence fragments according to the sequence analysis, and conjugate with an ovalbumin protein (OVA) as carrier protein using commercial services.
    1. Add hydrophobic amino acids (*e.g.*, methionine) to the N-terminal of antigenic reactive site for preventing the peptide from decomposition (*e.g.*, M-KASEDLKKHG).
    2. Lengthen the C-terminal of antigenic reactive site for exposing the core antigenic site to the immunocyte. Furthermore, conjugate the peptide with OVA by adding a cysteine (Cys, C) to the C-terminal (*e.g.*, M-KASEDLKKHG-NTVL-C).
5. Emulsify complete Freund's adjuvant for immunogen 1 or incomplete Freund's adjuvant for immunogen 2 with an equal volume of each synthetic peptide in PBS (3 ml, final concentration 30-50 µg/100 µl).
6. Inoculate immunogen 1 (0.1 mg) subcutaneously into each of 5 female BALB/c mice.
7. Perform subcutaneous booster injections five times using immunogen 2 at two-week intervals and collect test sera by tail clip sampling from the mice before every booster.
8. Determine sera titer for the first screening.
    1. Dissolve 100 µg of free peptide in 25 ml of reaction buffer and aliquot 50 µl of the solution into each well of a 96-well plate. Add 10 µl coupling reagent solution into each well and mix the plate. Incubate the plate for 2 hr at room temperature.
    2. Remove the contents of the well, wash each well 3 times with distilled water, and block the plate by adding 200 µl of blocking solution. Incubate for 1 hr at room temperature. Remove the contents and wash each well 3 times with distilled water.

3. Perform indirect ELISA (Protocol 5.1-5.7) for determining sera titer for the first screening. Choose the mouse with the highest titer (highest optical density) for spleen collection.

9. Three days prior to spleen collection, inoculate 0.1 mg of immunogen 1 subcutaneously into the mouse presenting the highest titer.
10. Collect the spleen cells from the selected immunized mice and fuse with murine myeloma cells F0 (sp2/0-Ag14) to obtain hybridoma cells for mAb generation[18].
11. 14 days after fusion, select the positive clones by screening the reactivity of hybridoma supernatants toward free synthetic peptide using indirect ELISA (Protocol 5.1-5.7).
12. Dilute the cells to an appropriate number per well for maximizing the proportion of wells that contain only one single clone (dilution cloning).
    1. Add 100 µl of cell culture medium to all the wells in the 96-well plate except well A1 which is left empty.
    2. Add 200 µl of the cell suspension to well A1. Then quickly transfer 100 µl from A1 to B1 and mix by gently pipetting. Repeat these 1:2 dilutions down the entire column, and then discard 100 µl from H1 so that it ends up with the same volume as the wells above it.
    3. Add an additional 100 µl of medium to column 1 with an 8-channel micropipette. Then quickly transfer 100 µl from each of the wells in column 1 to those in column 2 using the same pipette, and mix by gently pipetting.
    4. Using the same tips, repeat these 1:2 dilutions across the entire plate. Discard 100 µl from each of the wells in the last column.
    5. Bring the final volume of all wells to 200 µl by adding 100 µl medium to each well. Incubate plate undisturbed at 37 °C in a humidified CO_2 incubator.
    6. Check each well and mark all wells that contain just a single colony. Carry out two or more clonings until >90% of the wells containing single clones are positive for antibody production.
13. Screen the clones by western blot and dot blot (protocol 3.1-4.6). Then subculture colonies from the wells into larger vessels to expand cells for obtaining mAb. Usually each clone is transferred into a single well in a 12- or 24-well plate.
14. Measure the affinity between the mAbs and the muscle extracts from cow, goat, pig, dog, rabbit, tuna, chicken, seal, and four representative cetacean species by western blot and dot blot (protocol 3.1-4.6).
15. Inoculate selected hybridoma cells (up to 3 x 10^6) intraperitoneally into mice to induce ascites. Abdominal swelling is typically apparent within 7-10 days post hybridoma injection. Collect fluid using a hypodermic needle (less than 20 gauge).
16. Centrifuge ascites fluid (10,000 x g for 10 min) to remove cells and debris. Filter through a 0.45 µm filter. Add 1 to 20 ml of the sample, 15 ml binding buffer, and 3 to 5 ml of elution buffer into the protein G Sepharose column. Collect the elution fraction containing purified antibody from the mice ascites fluid.
17. Determine the antibody isotype by antibody isotyping kit using manufacturer's instructions.

3. Western Blot

1. Prepare 2x loading buffer containing β-mercaptoethanol (BME) by mixing 950 µl of 2x Laemmli sample buffer with 50 µl of BME. Dilute the muscle supernatants (protocol 1.1-1.4) in loading buffer with appropriate ratio for obtaining good signals: 1:50 (cetaceans and seal) and 1:5 (domestic animals and tuna) when using polyclonal rabbit anti-human Mb antibody, and 1:1 (pig, rabbit, chicken and tuna), 1:5 (cow, goat and dog), and 1:25 (cetaceans and seal) when antibody is from hybridoma supernatant.
2. Heat sample at 95 °C for 5 min. Load samples into the wells of SDS-PAGE gel (4% acrylamide stacking and 12% acrylamide separating) along with molecular weight markers. Run the gel for 5 min at 50 V then increase the voltage to 150 V to finish the run in about 1 hr.
3. Place the gel in 1x transfer buffer for 15 min. Transfer the separated protein to nitrocellulose (NC) membranes after they are separated by PAGE. Transfer can be done at 100 V for 60-90 min.
4. Prepare blocking solution: 1x PBS containing 0.1% Tween 20 with 5% nonfat dry milk. Block the NC membrane in 25 ml of blocking solution at room temperature for 1 hr. Wash three times for 5 min each with 15 ml phosphate-buffered saline with Tween 20 (PBST).
5. Incubate the membrane and primary antibody (ascites fluid or hybridoma supernatant) in 10 ml antibody dilution buffer diluted with 5% blocking solution at 4 °C overnight.
6. Wash the membrane three times again with PBST to clean off excessive antibody.
7. Incubate the membrane with alkaline phosphatase-conjugated goat anti-mouse IgG at 1:1,250 in blocker solution with gentle agitation for 1 hr at room temperature.
8. Wash the membrane again and incubate it in the 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride (BCIP/NBT) phosphatase substrate mixture for 10 to 20 min until color development.
9. Stop the reaction by washing the membrane in several changes of distilled water.

4. Dot Blot

1. Dilute the muscle supernatants (protocol 1.1-1.4) in 5% bovine serum albumin (BSA) in PBST with appropriate ratio for obtaining good signals: 1:5 for domestic animals and tuna, and 1:25 for cetaceans and seal. Spot 5 µl of samples onto membrane. Minimize the area that the solution penetrates (usually 3-4 mm diameter) by applying it slowly.
2. Dry the membrane at room temperature (*e.g.,* in a laminar flow for 30-60 min), block it with blocking solution in Petri dish for 1 hr at room temperature, and wash it with PBST.
3. Incubate the membrane with primary antibody (mAb from hybridoma supernatant at 1:10,000 or ascites fluid at 1:100,000 diluted in 5% blocking solution) for 1 hr at room temperature.
4. Use PBST to wash the membrane three times for 5 min each to remove excess antibody, and then incubate it with secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG at 1:1,250 in 5% blocking solution) for 1 hr at room temperature.
5. Wash the membrane again and incubate it in the BCIP/NBT phosphatase substrate mixture within 10 to 20 min until color development.
6. Stop the reaction by washing the membrane in several changes of distilled water.

5. Indirect ELISA

1. Prepare washing buffer (0.002 M imidazole buffered saline with 0.02% Tween 20). Wash the plate 3 times with washing buffer between each following step (protocol 5.2-5.5).
2. Prepare 1:25 dilution of muscle supernatants (protocol 1.1-1.4) in coating buffer. Coat a 96-well ELISA plate with 100 µl diluted supernatants at 4 °C overnight and block it with blocking buffer (1% BSA in PBS) for 1 hr at room temperature.
3. Prepare 1:2,000 dilution of the purified mAb with diluted buffer and add 100 µl of diluted mAb to each well. Incubate the plate for 1 hr at room temperature.
4. Add goat anti-mouse IgG conjugated with horseradish peroxidase (1:200 dilution in diluted buffer) for further incubation.
5. Add peroxidase substrate to each well (100 µl/well) and incubate for 10-15 min.
6. Stop the enzyme reaction by the peroxidase stop solution (100 µl/well) when color development is observed.
7. Read the optical density at 450 nm using a microplate spectrophotometer.

6. Preparation of Colloidal Gold-labeled mAb

Note: The color of colloidal gold solution and the mixture should always be red. Adjust pH, concentration of mAb, centrifuge speed when black precipitate is noticed. Steps 6.1 and 6.2 are optimization steps.

1. Add purified detecting mAb (50 µg/ml, 3 µl) to 100 µl of colloidal gold solution with pH values varying from 5-9. The minimum pH that keeps the color red for two hours is considered as the optimum pH. Note: In this study, 0.1 M potassium carbonate was used to adjust colloid gold (40 nm) solution to pH 8.0 (optimum pH).
2. Add various amount of purified detecting mAb (500 µg/ml, 1-20 µl) to 100 µl of colloidal gold solution at pH 8.0. Note: The optimum concentration in this study is 6 µg/ml (no black precipitate).
3. According to the above results, add 60 µg of purified detecting mAb drop-wise to 10 ml of colloid gold solution. Emulsify the mixture gently at room temperature for 10 min. Add 2 ml of 5% BSA solution in PBS (pH 7.4) to the mixture and emulsify gently at room temperature for 15 min to reduce background interference.
4. Centrifuge the mixture at 10,000 x g for 30 min at 4 °C.
5. Remove the supernatant with unconjugated antibody carefully and suspend the resulting pellets in 4 ml PBST containing 1% BSA and 0.1% Tween 20, and repeat centrifugation and suspension several times.
6. Suspend the final precipitates in 1 ml PBST and store it at 4 °C until used.

7. Construction of Immune Strip

Note: **Figure 1** shows the immune strip design. Prepare and assemble the strips in a low-humidity laboratory environmental condition (< 20% Relative Humidity) for prolonged storage life (> 1 yr). The dimensions of pads and membrane are: conjugate pad 300 mm x 10 mm, absorbent pad 300 mm x 24 mm, sample pad 300 mm x 24 mm, NC membrane 300 mm x 25 mm, pasteboard 300 mm x 80 mm.

1. Add colloid gold-labeled mAb solution from step 6.6 with a micropipette to saturate the conjugate pad and then dry it at 37 °C for 1 hr before assembling.
2. Distribute the specific antigen-capturing mAb (500 µg/ml) on the test zone, and rabbit anti-mouse IgG (500 µg/ml) on the control zone for detecting mAb on the NC membrane using a pipette or immunostrip printer. Maintain distance (>5 mm) between the two zones to avoid interference.
3. Paste the conjugated pad, absorbent pad and NC membrane on the pasteboard with double-sided tape.
   Note: Overlap the pads on each side of the NC membrane by about 2 mm. Inappropriate strip construction will result in an incomplete test.
4. Place the sample pad over the conjugate pad (2 mm) and paste it on the pasteboard.
5. Create 6-mm-wide strips with a paper cutter. Pack the strips in the aluminum foil bag with desiccant, and store them at 4 °C until used.

8. Cross-reactivity Test

1. Homogenize 0.03 g of raw muscle sample with 1 ml PBS (containing 0.1% BSA) in a 1.5 ml centrifuge tube using a bamboo stick or grinding rod.
2. Hold the strip by the end opposite to the test areas and dip the sample pad part into the specimen for 5-10 min and observe the result directly.
   1. Optional: Collect 500 µl supernatant and transfer it to a new centrifuge tube.
      Note: This step is suggested if the signal is not obvious when the strip is directly soaked into the supernatant.
3. Test various muscle samples in triplicate.
   Note: Here we tested tuna, chicken, seal, 15 species of cetaceans and 5 species of terrestrial mammals.
4. Have five independent inspectors repeat 8.3 for five times, *i.e.*, use 575 strips in total.

Representative Results

Monoclonal antibody characteristics

We developed two IgG_1 mAbs (CGF5H9 and CSF1H13) recognizing two synthetic peptides (MKASEDLKKHGNTVLC and AIIHVLHSRHPAEFGC), respectively, of cetacean Mb, and these were used to construct a sandwich-type colloidal gold immunochromatographic test strip for the rapid detection of cetacean Mb. **Figure 2** shows that CGF5H9 detects cetaceans and other mammals as a single stained band at a predicted molecular weight of approximate 17 kDa. The common minke whale (*Balaenoptera acutorostrata*) shows a comparatively fainter

band than the bands of other cetaceans. Bands are absent for tuna, chickens and seals, whereas a band at about 50 kDa is observed for pigs. Although there are multiple nonspecific bands for pigs and tuna, CSF1H13 is highly specific because it reacts only with cetaceans and seals as a band at a predicted molecular weight of approximate 17 kDa. Minke whale only shows a strong signal at 1:10 (data not shown) with no signal observed at 1:25. **Figure 2** shows identical results in dot blot.

Figure 3A shows that CGF5H9 demonstrates positive signal for cetaceans, rabbits, dogs, goats, and cows (OD value > 3.0); weak positive signal for pigs (OD value = 1.5); negative signal for seals, chickens and tuna. **Figure 3B** shows that CSF1H13 expresses high affinity towards cetaceans and seals (OD value > 3.0). Both CSF1H13 and CGF5H9 can react strongly with all four cetacean species. These four species are from different families (minke whale: Balaenopteridae; bottlenose dolphin: Delphinidae; dwarf sperm whale: Kogiidae; finless porpoise: Phocoenidae), indicating the broad reactivity to diverse cetacean species.

For strip construction, CGF5H9, which recognizes the Mbs of cetaceans and other mammals, is colloid gold-labeled and used as the detection antibody to bind myoglobin, and CSF1H13 is coated on the test line only to capture the Mbs of cetaceans and seals. Therefore the test line is designed to show a positive signal when both mAbs detect cetacean Mb. Because the Mb from non-cetacean animals can only be detected by one of these two mAbs, the test line shows a negative result when muscle samples from other animals are tested. The control line always shows a positive result because rabbit anti-mouse IgG binds colloid gold-labeled CGF5H9. A failed result in the control line indicates that the quality of the materials on the strip is poor.

Strip test

Figure 4 shows the signal bands at both test line and control line when cetacean muscle samples are used. When the sample is not from cetaceans, there is only a single band at the control line with the absence of band at the test line. A successful result can be observed directly in 5-10 min after homogenizing 0.03 g of muscle with 10 ml PBS containing 0.1% BSA using a plastic or bamboo stick and soaking the strip into the mixture. After testing 15 cetacean species and eight non-cetacean species in triplicate, specificity (the percentage of non-cetacean samples correctly identified) and sensitivity (the percentage of cetacean samples correctly identified) are both 100%.

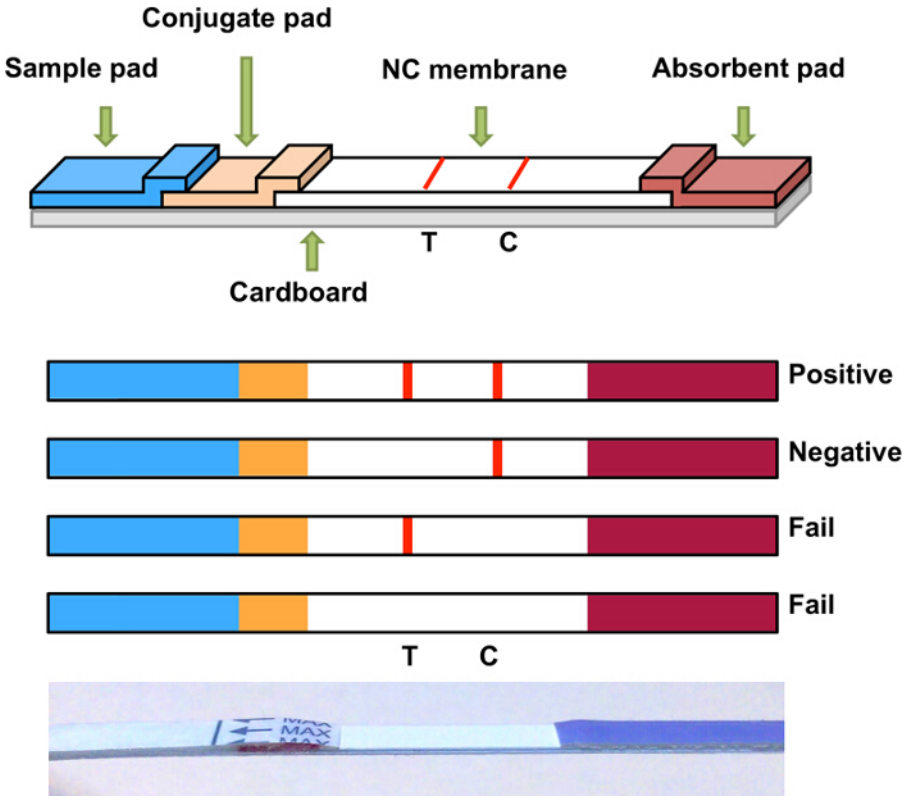

Figure 1. Design of the immune strip. All the components are carefully layered on to the plastic backing card so that they overlap. This allows the reagents and sample flow up through the membrane and to the absorbent pad. T: test zone. C: control zone. This figure has been reproduced from Lo, C. *et al.* Rapid immune colloidal gold strip for cetacean meat restraining illegal trade and consumption: implications for conservation and public health. *PLoS ONE* **8**, e60704 (2013). doi:10.1371/journal.pone.0060704. Please click here to view a larger version of this figure.

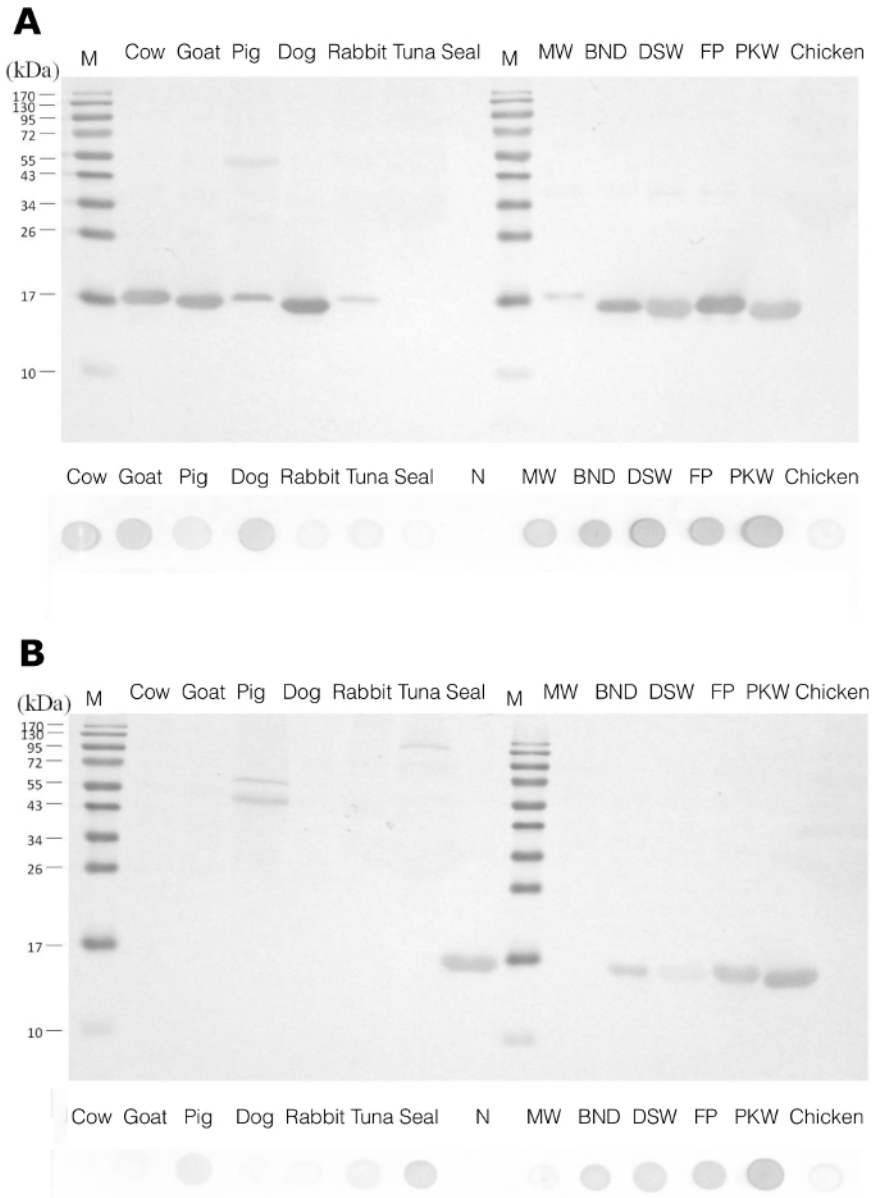

Figure 2. Western blot and dot blot analysis using the hybridoma supernatants. (**A**) CGF5H9, (**B**) CSF1H13. Both hybridoma supernatants can detect cetaceans as a single stained band at a predicted molecular weight of approximate 17 kDa. MW: minke whale, BND: bottlenose dolphin, DSW: dwarf sperm whale, FP: finless porpoise, PKW: pygmy killer whale, N: PBS (negative control). This figure has been reproduced from Lo, C. *et al.* Rapid immune colloidal gold strip for cetacean meat restraining illegal trade and consumption: implications for conservation and public health. *PLoS ONE* **8**, e60704 (2013). doi:10.1371/journal.pone.0060704. Please click here to view a larger version of this figure.

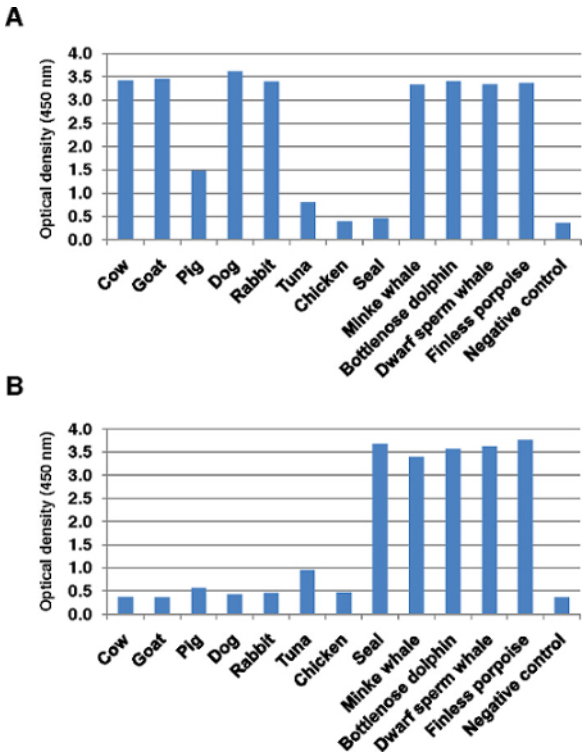

Figure 3. Indirect ELISA of muscle extracts of different species using purified mAbs. (**A**) CGF5H9, (**B**) CSF1H13. Only cetaceans can produce strong positive signals in both mAbs. This figure has been reproduced from Lo, C. *et al.* Rapid immune colloidal gold strip for cetacean meat restraining illegal trade and consumption: implications for conservation and public health. *PLoS ONE* **8**, e60704 (2013). doi:10.1371/journal.pone.0060704. Please click here to view a larger version of this figure.

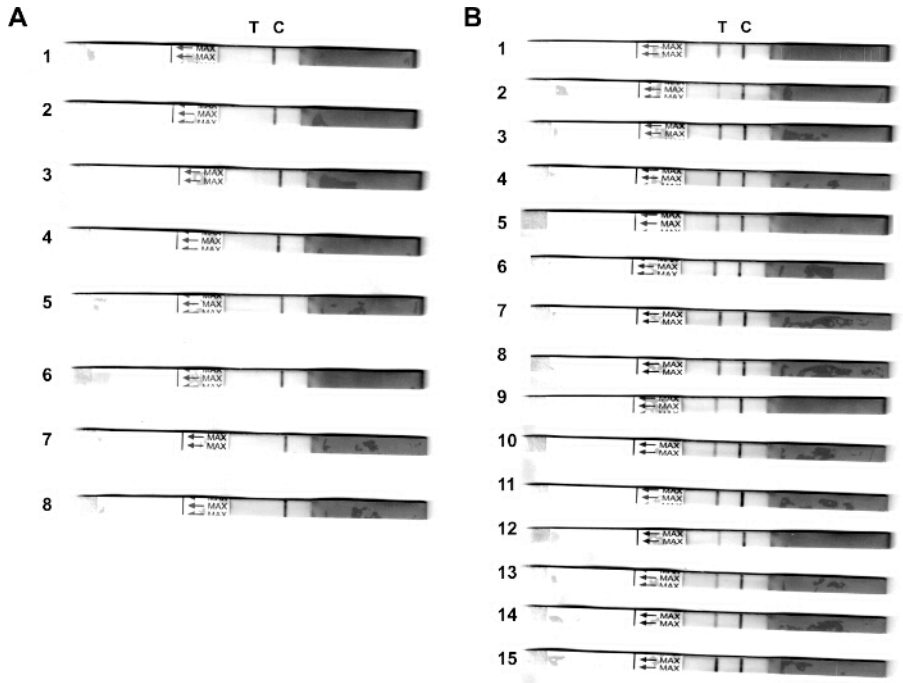

Figure 4. Specificity of the immune colloidal gold strip. T: test line. C: control line. Only when cetacean muscle samples are used, successful results (signal bands at both test line and control line) can be observed. (**A**) Non-cetacean samples: 1: Cow. 2: Goat. 3: Pig. 4: Dog. 5: Rabbit. 6: Tuna. 7: Chicken. 8: Harbor seal (*Phoca vitulina*). (**B**) Cetacean samples: 1: Common minke whale (*Balaenoptera acutorostrata*). 2: Omura's whale (*Balaenoptera omurai*). 3: Bottlenose dolphin (*Tursiops aduncus*). 4: Bottlenose dolphin (*T. truncatus*). 5: Fraser's dolphin (*Lagenodelphis hosei*). 6: Indo-Pacific humpback dolphin (*Sousa chinensis*). 7: Risso's dolphin (*Grampus griseus*). 8: Pantropical spotted dolphin (*Stenella attenuata*). 9: Rough-toothed dolphin (*Steno bredanensis*). 10: Pygmy killer whale (*Feresa attenuata*). 11: Short-finned pilot whale (*Globicephala macrorhynchus*). 12: Melon-headed whale (*Peponocephala electra*). 13: Dwarf sperm whale (*Kogia sima*). 14: Pygmy sperm whale (*K. breviceps*). 15: Finless porpoise (*Neophocaena phocaenoides*). This figure has been reproduced from Lo, C. *et al.* Rapid immune colloidal gold strip for cetacean meat restraining illegal trade and consumption: implications for conservation and public health. *PLoS ONE* **8**, e60704 (2013). doi:10.1371/journal.pone.0060704. Please click here to view a larger version of this figure.

Cetacean species	**Non-cetacean species**
Pygmy sperm whale (*Kogia breviceps*)	Harbor seal (*Phoca vitulina*)
Dwarf sperm whale (*Kogia sima*)	Dog (*Canis lupus familiaris*)
Short-finned pilot whale (*Globicephala macrorhynchus*)	Rabbit (*Oryctolagus cuniculus*)
Melon-headed whale (*Peponocephala electra*)	Pig (*Sus scrofa*)
Pygmy killer whale (*Feresa attenuata*)	Goat (*Capra hircus*)
Pantropical spotted dolphin (*Stenella attenuata)*	Cattle (*Bos Taurus*)
Bottlenose dolphin (*Tursiops truncatus*)	Chicken (*Gallus gallus*)
Bottlenose dolphin (*Tursiops aduncus*)	Yellowfin tuna (*Thunnus albacares*)
Fraser's dolphin (*Lagenodelphis hosei*)	
Indo-Pacific humpback dolphin (*Sousa chinensis*)	
Rough-toothed dolphin (*Steno bredanensis*)	
Risso's dolphin (*Grampus griseus*)	
Finless porpoise (*Neophocaena phocaenoides*)	
Common minke whale (*Balaenoptera acutorostrata*)	
Omura's whale (*Balaenoptera omura*i)	

Table 1. The species from which muscle was collected and tested in this study. Species include tuna, chicken, seal, 5 species of terrestrial mammals, and 15 species of cetaceans (4 families).

Species	Accession no.
Common minke whale (*Balaenoptera acutorostrata*)	P02179
Pygmy Bryde's whale (*Balaenoptera edeni*)	Q0KIY2
Humpback whale (*Megaptera novaeangliae*)	P02178
Gray whale (*Eschrichtius robustus*)	P02177
Sperm whale (*Physeter macrocephalus*)	P02185
Pygmy sperm whale (*Kogia breviceps*)	Q0KIY5
Dwarf sperm whale (*Kogia sima*)	P02184
Short-beaked common dolphin (*Delphinus delphis*)	P68276
Long-finned pilot whale (*Globicephala melas*)	P02174
Killer whale (*Orcinus orca*)	P02173
Melon-headed whale (*Peponocephala electra*)	Q0KIY3
Pantropical spotted dolphin (*Stenella attenuata*)	Q0KIY6
Bottlenose dolphin (*Tursiops truncatus*)	P68279
Harbor porpoise (*Phocoena phocoena*)	P68278
Amazon river dolphin (*Inia geoffrensis*)	P02181
Longman's beaked whale *(Indopacetus pacificus*)	Q0KIY9
Hubbs' beaked whale (*Mesoplodon carlhubbsi*)	P02183
Cuvier's beaked whale (*Ziphius cavirostris*)	P02182
Harbor seal (*Phoca vitulina*)	P68080
Cattle (*Bos Taurus*)	P02192
Goat (*Capra hircus*)	B7U9B5.3
Horse (*Equus caballus*)	P68082
Pig (*Sus scrofa*)	P02189
Dog (*Canis lupus familiaris*)	P63113
Chicken (*Gallus gallus*)	P02197
Ostrich (*Struthio camelus*)	P85077
Yellowfin tuna (*Thunnus albacares*)	P02205

Table 2. Myoglobin sequences used in this study with respective GenBank accession numbers. Species include tuna, chicken, ostrich, domestic mammals, seal and 18 species of cetaceans (7 families). This table has been reproduced from Lo, C. *et al.* Rapid immune colloidal gold strip for cetacean meat restraining illegal trade and consumption: implications for conservation and public health. *PLoS ONE* **8**, e60704 (2013). doi:10.1371/journal.pone.0060704.

Discussion

Using a synthetic peptide conjugated to carrier protein is remarkably more effective compared to its cognate protein. For a sandwich-based technique, because the mAb is developed using epitopes with known relative locations, the two mAbs in this study are not likely to interfere with each other's interaction with the target antigen epitope. Moreover, the reactivity between the native protein and the antibody of mice immunized with the synthetic peptide-conjugate may be stronger than the reactivity between the native protein and the antibody produced from the native protein[19]. The use of synthetic peptide conjugates is therefore recommended for effective immunization procedures and generation of appropriate anti-peptide mAb.

The structure of a protein mainly involves the sequence of amino acids in the polypeptide chain. Each amino acid has its side-chain leading to specific properties, and slightly changing the amino acid sequence results in structure changes. Because peptides with a length of 10-20 amino acids are ideal for antibody preparation, the length of the synthetic peptides (immunogen) at the C-terminal region was increased to ensure that the core antigenic region would be recognized. Therefore, the amino acid residues among Mbs of various animals resulting in different epitope structures could be efficiently differentiated. For example, **Figure 3A** shows the mentioned peptide design contributes to CGF5H9 reacting strongly with cetaceans but negatively with seals. Another example is the distinct affinities toward chickens and dogs of CGF5H9 although the chicken has an identical sequence to that of the dog in the core antigenic site 2. This indicates that the sequence difference in the outer region could lead to the structure change and thus variable binding affinity between antigen and antibody.

Western blot, dot blot, and indirect ELISA were used in our method for screening suitable mAbs. Western blot is widely used to detect specific proteins in tissue extract or homogenate. In this technique, gel electrophoresis is used to separate denatured proteins by the length of the polypeptide. Therefore, it is possible to confirm if the signal indicates the predicted protein molecular weight. However, the detection result

(positive or negative, strong or weak signal) may not have represented the real situation of antigen-antibody binding because denatured proteins are used. Consequently, dot blot can be used for second-stage screening. Dot blot is a technique for detecting proteins. It represents a simplification of western blot method. In dot blot, a mixture containing the molecule to be detected is applied directly on a membrane as a dot. This differs from a western blot because protein samples are not denatured. Note that this technique offers no information on the size of the target biomolecule, and a single dot will appear if two molecules of different sizes are detected. Finally, indirect ELISA is used for a ligand-binding assay in order to generate a signal that can be properly quantified. It provides more information of mAb characteristics and thereby facilitates the strip construction.

Concentrations of Mb in the muscle are variable depending on the collection location. For example, swimming muscles (axial muscles) in cetaceans have a significantly higher content of Mb compared with non-swimming muscles, and samples from young cetaceans would have lower Mb concentration because the Mb concentration increases throughout an animal's life[20]. Initially, CSF1H13, which only captures the Mb of cetaceans and seals, was intended to be colloid gold-labeled and be detecting antibody, and CGF5H9, which recognizes the Mb of many species, would be capture antibody on the test line. We hypothesized that the detecting antibody should be more specific and the capture antibody should be more general. However, a weak positive signal was found on the test line when cetacean samples with low Mb concentrations were used (data not shown). The problem was resolved when the positions of the two mAbs were reversed as described in the representative results. A good signal was even shown for a newborn cetacean (a stranded Omura's whale) (**Figure 4**). It is unclear whether the characteristics and concentration of mAbs contribute to this phenomenon.

In this study, frozen-thawed muscle samples homogenized with PBS were used on the strip test. Other sample conditions and preparation methods may affect the result. For example, salt-soluble protein such as Mb should be extracted using PBS rather than pure water. Otherwise, the extraction may be inadequate, which could lead to an aberrant result. The appropriate extraction buffer: meat sample ratio is partly responsible for the successful interpretation of the strip result. Large amount (0.3 g in 1 ml buffer) of control samples (*e.g.,* domestic animals) could cause positive result and blurred background. However, the ratio used in this study (1: 0.03) produced correct results. Only fresh muscle samples can be used for this strip test. Protein could be hydrolyzed or denatured after certain treatments (such as curing by soy sauce and boiling), which could cause positive results not only for cetacean muscle samples but also samples from other animals (data not shown). Therefore, it is suggested that variable sample sources and different construction design plans should be used during strip development.

In conclusion, this protocol describes the development of two mAbs strongly reactive to the Mb of cetaceans, and these mAbs are applied on a quick test strip to differentiate the Mb of cetaceans from seal and other animals. Although reliable PCR-based DNA analysis for the identification of cetacean meat is available[12], it is labor intensive and time consuming. The quick test strip is a dependable and rapid technique that can be used in the field to identify cetacean meats, which is highly desirable for regulatory agencies[21]. It is likely that the strip can be developed for detecting specific Mbs from animals such as horses or pigs.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Robards, M. D., & Reeves, R. R. The global extent and character of marine mammal consumption by humans: 1970-2009. *Biol. Conserv.* **144**, 2770-2786 (2011).
2. Booth, S., & Zeller, D. Mercury, food webs, and marine mammals: implications of diet and climate change for human health. *Environ. Health Perspect.* **113**, 521-526 (2005).
3. Endo, T. *et al.* Total mercury, methyl mercury, and selenium levels in the red meat of small cetaceans sold for human consumption in Japan. *Environ. Sci. Technol.* **39**, 5703-5708 (2005).
4. Tryland, M. *et al. Human pathogens in marine mammal meat.* Norwegian Scientific Committee for Food Safety (VKM) (2011).
5. Matsunaga, T. *et al.* A quick and simple method for the identification of meat species and meat products by PCR assay. *Meat Sci.* **51**, 143-148 (1999).
6. Dalebout, M. L., Helden, A. V., van Waerebeek, K., & Baker, C. S. Molecular genetic identification of southern hemisphere beaked whales (Cetacea: Ziphiidae). *Mol. Ecol.* **7**, 687-694 (1998).
7. Dalmasso, A. *et al.* A multiplex PCR assay for the identification of animal species in feedstuffs. *Mol. Cell. Probes* **18**, 81-87 (2004).
8. Kesmen, Z., Sahin, F., & Yetim, H. PCR assay for the identification of animal species in cooked sausages. *Meat Sci.* **77**, 649-653 (2007).
9. Liu, L., Chen, F.-C., Dorsey, J. L., & Hsieh, Y.-H. P. Sensitive Monoclonal Antibody-based Sandwich ELISA for the Detection of Porcine Skeletal Muscle in Meat and Feed Products. *J. Food Sci.* **71**, M1-M6 (2006).
10. Kotoura, S. *et al.* A Sandwich ELISA for the Determination of Beef Meat Content in Processed Foods. *Food Sci. Techno. Res.* **15**, 613-618 (2009).
11. Hsieh, Y. H., Chen, Y. T., & Gajewski, K. Monoclonal antibody-based sandwich ELISA for reliable identification of imported Pangasius catfish. *J. Food Sci.* **74**, C602-607 (2009).
12. Ross, H. A. *et al.* DNA surveillance: web-based molecular identification of whales, dolphins, and porpoises. *J. Hered.* **94**, 111-114 (2003).
13. Ngom, B., Guo, Y., Wang, X., & Bi, D. Development and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: a review. *Anal. Bioanal. Chem.* **397**, 1113-1135 (2010).

14. Muldoon, M. T., Onisk, D. V., Brown, M. C., & Stave, J. W. Targets and methods for the detection of processed animal proteins in animal feedstuffs. *Int. J. Food Sci. Technol.* **39**, 851-861 (2004).
15. Rao, Q., & Hsieh, Y. H. Evaluation of a commercial lateral flow feed test for rapid detection of beef and sheep content in raw and cooked meats. *Meat Sci.* **76**, 489-494 (2007).
16. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731-2739 (2011).
17. Atassi, M. Z. Antigenic structure of myoglobin: the complete immunochemical anatomy of a protein and conclusions relating to antigenic structures of proteins. *Immunochemistry.* **12**, 423-438 (1975).
18. Zhang, C. Hybridoma technology for the generation of monoclonal antibodies. *Methods Mol. Biol.* **901**, 117-35 (2012).
19. Kao, D. J., & Hodges, R. S. Advantages of a synthetic peptide immunogen over a protein immunogen in the development of an anti-pilus vaccine for Pseudomonas aeruginosa. *Chem. Biol. Drug Des.* **74**, 33-42 (2009).
20. Dolar, M. L., Suarez, P., Ponganis, P. J., & Kooyman, G. L. Myoglobin in pelagic small cetaceans. *J. Exp. Biol.* **202**, 227-236 (1999).
21. Lo, C. *et al.* Rapid immune colloidal gold strip for cetacean meat restraining illegal trade and consumption: implications for conservation and public health. *PLoS ONE* **8**, e60704 (2013).

