








Pet Amphibian Trade Pathogen Testing Study Instructions for sampling

1. Randomizing/selecting habitats to sample

<p>Step 1 Go to the random generator number app</p>  <p>https://brunnerlab.shinyapps.io/RandomNumGenerator/</p>	<p>Step 2 Fill up the form</p> <h3>Random Number Generator</h3> <p>Number of random numbers desired (number of habitats, up to 100)</p> <p>Enter here the number of habitats in your facility</p> <p>First habitat # in your numbering system:</p> <p>Enter here the first ID number of your habitats ID system</p> <p>Last habitat # in your numbering system:</p> <p>Enter here the last ID number of your habitats ID system</p> <p>Generate! ← When finished, click <u>Generate!</u></p>
<p>Step 3</p> <p>Sample the habitats <i>in the order of the generated list</i></p> <p>Copy-paste into another document or print the numerical sequence out</p>	
<p>If you have any questions about the protocol See the Instructional video here:</p>  <div style="display: flex; justify-content: space-around; align-items: center;">    </div>	



Pet Amphibian Trade Pathogen Testing Study Instructions for sampling

2. Swabbing individual animals from terrestrial habitats

Step 1

Fill out the label on the WhirlPak bag
(use a fine tip permanent marker)

4725 - 16	Swab Sample
Habitat ID: _____	Date: ____/____/____
Species: _____	
Stage(s): <input type="checkbox"/> Larval <input type="checkbox"/> Juvenile or adult	
Number of Individuals in Tank: _____	
Substrate in bottom of tank is:	
<input type="checkbox"/> Bark or fiber <input type="checkbox"/> Rock or gravel <input type="checkbox"/> Soil or dirt	
<input type="checkbox"/> Paper towel <input type="checkbox"/> Other: _____	
Structures in Tank:	
<input type="checkbox"/> Live plants <input type="checkbox"/> Artificial plants	
<input type="checkbox"/> Wood <input type="checkbox"/> Stones <input type="checkbox"/> Other: _____	

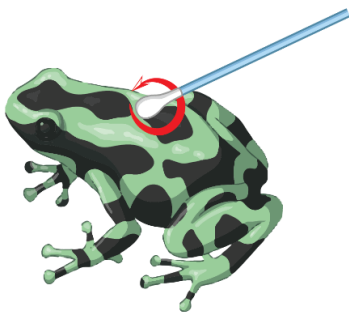
Step 2

Put on new gloves



Step 3

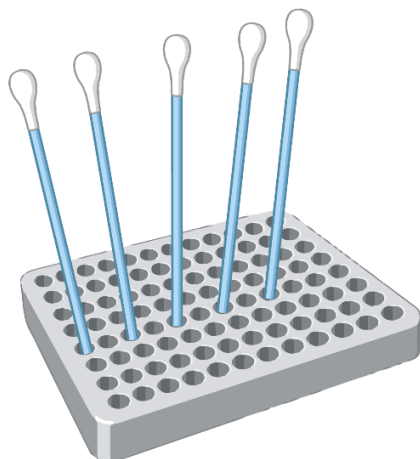
Swab the animals
1 swab per animal



Follow this pattern:

- Roll 5 times on the back (dorsal)
- Roll 5 times on the belly (ventral)
- Roll 5 times on each foot
- Roll 5 times on the cloaca area

Step 4
Place swab in the holder to air dry
(5 minutes*)



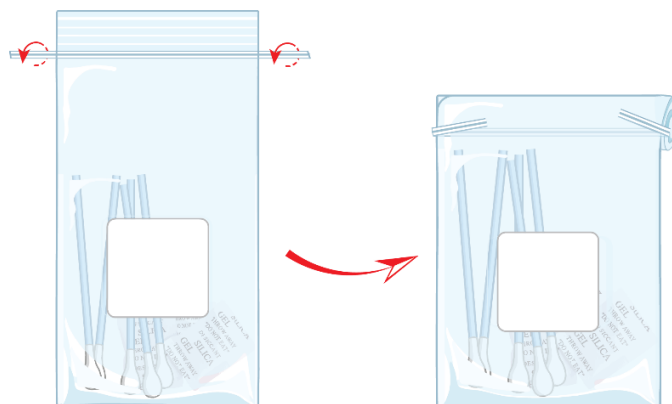
*This time is just a suggestion, and it could vary according to the local conditions of the sampling

Step 5

Repeat steps 3 and 4 until all animals from the habitat are swabbed (up to five individuals)

Step 6

- Place swabs in the WhirlPak bag
- Place 3 desiccant packs in the bag
- Roll up end of bag and twist the twist-tie to seal the bag



Step 7

Move on to the next habitat on the list and repeat steps 1-6

If you have any questions about the protocol
See the Instructional video here:



Pet Amphibian Trade Pathogen Testing Study Instructions for sampling

3. Sampling water from aquatic habitats

Step 1

Fill out the label on the WhirlPak bag
(use a fine tip permanent marker)

4725 - 1 Filter Sample

Habitat ID: _____ Date: ___/___/___

Species: _____

Stage(s): Larval Juvenile or adult

Number of individuals in tank: _____

Water volume in tank: _____

Is water recirculated? Yes No

If yes, within: One tank Multiple tanks

Is tank water filtered? Yes No

If yes, with: Biofilter/sponge Charcoal

UV Other: _____

Volume filtered for sample (mL): _____

Step 2

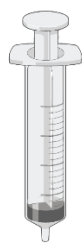
Put on new gloves



Step 3

On a clean surface organize:

1. Unwrap one syringe
2. Unwrap one filter
3. Separate one red rubber syringe stopper
4. Unwrap one sealed red luer lock cap
5. 3 ml syringe with ATL
6. A bowl (not provided) to discard water



1



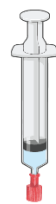
2



3



4



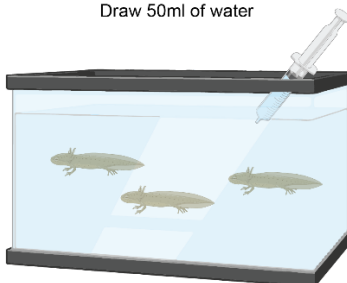
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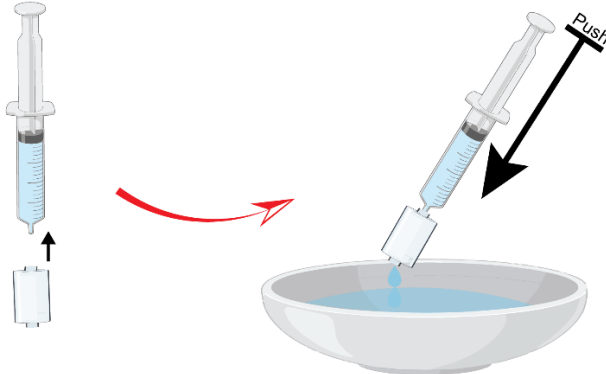
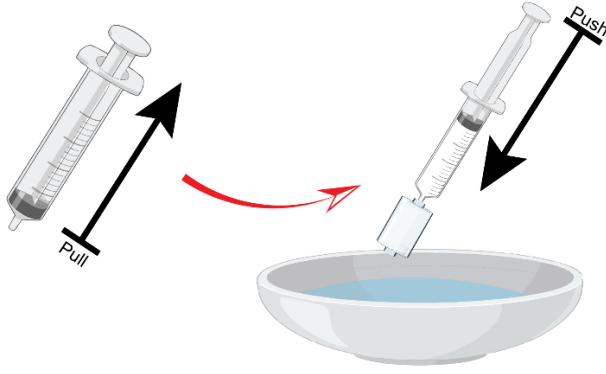

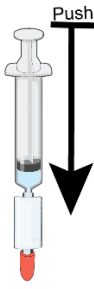


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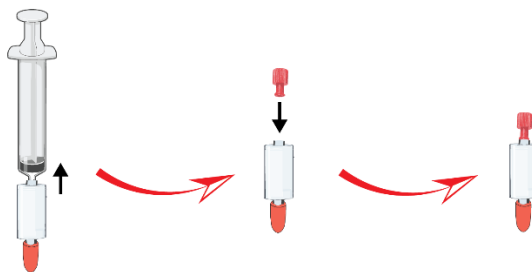
Step 4

Draw 50ml of water

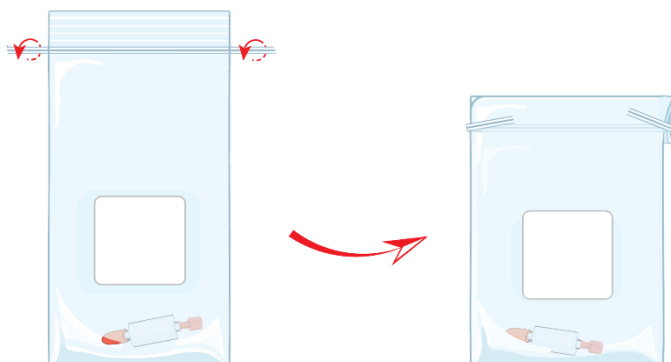


<p>Step 5 Screw filter onto filled syringe Push water through filter on top of the bowl Discard the excess of the water</p> 	
<p>Step 6 Repeat steps 4 and 5*</p> <p>*If water becomes very difficult to press through the filter, the filter is likely clogged</p> <p>Write the volume of water filtered on the label</p>	
<p>Step 7 Unscrew filter Draw 50 ml of air Screw filter and push air through filter</p> 	
<p>Repeat step 7 one more time Remove the filter from syringe Tap the filter outlet on a clean paper</p>	
<p>Step 8 Take the red syringe stopper Press it over the filter's outlet Unscrew the red luer cap from the 3 ml syringe Attach the filter into the 3 ml syringe</p> 	<p>Step 9 Push liquid from the 3 ml syringe into the filter Unscrew 3ml syringe from filter Screw a red luer lock cap into the filter</p> 

Step 10
Unscrew 3ml syringe from filter
Screw a red luer lock cap into the filter



Step 11
Place filled, sealed, filter into labelled WhirlPak bag
Roll up end of the bag and twist twist-ties at ends



Step 12

Move on to next habitat and repeat steps 1 to 11

If you have any questions about the protocol
See the Instructional video here:



Preparing for shipping

- Create and write down a unique PIN (4-8 digits is fine) on the included sheet
 - Keep one copy and send the other to us
 - This is the only way to retrieve your results, so please be sure not to lose the PIN
- Ensure all bags are closed and *labelled*
- Place all bagged samples in padded return envelope
- Make sure return label is firmly affixed
- Place in any outgoing mail drop

Questions?

- You can ask questions or obtain additional supplies by contacting Ashley Brinkman at Ashley@petadvocacy.org or (202) 452-1525 extension 1010

Thank you!

Pet Advocacy Network Pet Amphibian Trade Pathogen Testing Study

Detailed instructions for sampling

Randomizing/selecting habitats to sample

Instructional video here:



<https://youtu.be/CeCwUUq7xm4>

Rational: We want to get an unbiased, representative sample from your facility. We therefore need to make sure that a random selection of habitats are sampled. Habitats are defined as tanks, containers, or groups of housing containers between which water or animals can move.

1. Go to: <https://brunnerlab.shinyapps.io/RandomNumGenerator/>
2. Enter the number of habitats in your facility (and optionally, the starting and ending number, if you already have a numbering scheme)
 - It does not matter how *you* number your habitats, just so that they are numbered in some sequential way
3. Click “Generate!”, and then print out the list
4. Sample the tanks *in the order of this list*
 - You will likely not get to the end of the list with the materials we have provided. That is OK. Just go until you run out of materials or you can’t stand this exercise any more... We’ll take whatever you can give
 - You may run out of swabs before you run out of filters, or vice versa. This is OK, too. Just move on to the next tank in the list and sample it if you can

Swabbing individual animals from *terrestrial* habitats

Instructional video here:



<https://youtu.be/cye9x4a-zJs>

Overview: We ask you to swab the skin of *up to* five individuals per habitat. If there is one animal, swab that one. If there are 30, sample the first five you catch. If you run out of supplies, sample as many as you can.

1. Wear new gloves for each habitat. You do not need to change gloves between individuals within a habitat
 - If you have sweaty or clammy hands, you may wish to double-glove to make it easier to change the outer pair of gloves between tanks
2. Fill out the label on the WhirlPak bag (**please use a fine tip permanent marker as pens will smudge**) (**Figure 1**)
 - Habitat ID (to relate results back to a habitat)
 - Species (Latin binomial, please)
 - Life stage(s) (Larva vs. adult)
 - Number of individuals in the tank
 - Approximate surface area of tank bottom
 - *Please provide units!* (Metric is preferred, but we can convert)
 - Type of substrate in the tank (bark or fiber, soil or dirt, paper towel, or other)

4725 - 16 Swab Sample
Habitat ID: 1 Date: 8/30/2021
Species:
Ambystoma mavortium
Stage(s): Larval Juvenile or adult
of Individuals in Tank: 1
Surface area of tank bottom: 500cm²
Substrate in bottom of tank is:
 Bark or fiber Rock or gravel Soil or dirt
 Paper towel Other: _____
Structures in Tank:
 Live plants Artificial plants
 Wood Stones Other: None

Figure 1. Example label for swab

3. Swab the first animal
 - Open the swab
 - Hold the animal firmly so that it will not escape
 - It can be helpful to place the animal in a sandwich bag, as seen in *Figure 2*, to limit its movement. If you decide to do this, use a clean bag for each animal.

- Run the swab along the animal's skin, pushing enough to bend the swab-handle
 - Roll the swab *against* the direction of movement
- Follow this pattern (Figure 2):
 - 5 times on dorsal surface/back
 - 5 times on ventral surface/belly
 - 5 times on each foot
 - 5 times on inguinal region/drink patch/around the cloaca
- Place swab in the holder to air dry for a few minutes (Figure 3)



Figure 2. Salamander in bag being swabbed

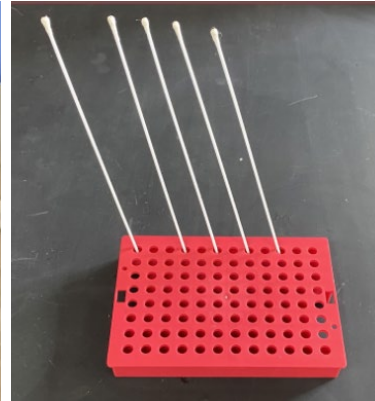


Figure 3. Example of swabs drying in provided rack. Make sure that swabs from different tanks don't touch each other if

4. Repeat until all animals are swabbed (up to five individuals)
 - Do not sample the same animal twice, please. Set aside those animals you have swabbed until you are finished
5. Once the last swab is air dried for a few minutes, place all swabs in the labelled WhirlPak bag, swab side down (away from the opening)
6. Add three desiccant packs
7. Roll up end of bag and twist twist-ties at ends to seal the bag
8. Move on to the next tank or container on the list
 - Be sure to change gloves

Sampling water from *aquatic* habitats

Instructional video here:



<https://youtu.be/uMAYfNVY8sA>

Overview: We want to collect DNA-bearing materials (skin sheds, mucous, crud, etc.) from the water housing animals (eDNA). We want a single eDNA sample per tank. Filtering more water is “better,” but please do not break the filter (or your fingers!) trying to push too much through. We will take what we can get. That being said, please tell us how much water was pushed through the filter and the overall volume in the tank.

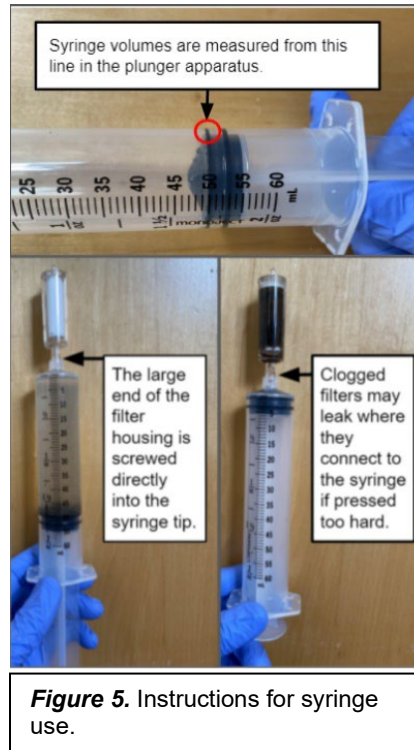
1. Wear new gloves for each habitat
 - If you have sweaty or clammy hands, you may wish to double-glove to make it easier to change the outer pair of gloves between habitats
2. Fill out the label on the WhirlPak bag (**please use a fine tip permanent marker as pens will smudge**) (**Figure 1**)
 - Habitat ID (to relate results back to a habitat)
 - Species (Latin binomial, please)
 - Life stage(s) (Larva vs. adult)
 - Number of individuals in the tank
 - Approximate volume of water in tank
 - *Please provide units!* (Metric is preferred, but we can convert)
 - *Leave “Volume filtered (mL)” blank until after filtering*

4725 - 1 *Filter Sample*
Habitat ID: 7 Date: 8/30/2021
Species: Agalychnis callidryas
Stage(s): Larval Juvenile or adult
of individuals in tank: 12
Water volume in tank: 40L
Is water recirculated? yes no
If yes, within: One tank Multiple tanks
Is tank water filtered? yes no
If yes, with: Biofilter/sponge Charcoal
 UV Other: _____
Volume filtered for sample (mL): 100mL

Figure 4. Example label for filter

3. Open large (60 mL) syringe and filter from wrappers
 - keep the filter wrapper so you have a clean spot to place the filter
4. Filter water
 - Draw up 50 mL water (use gradations on syringe)

- Screw filter onto filled syringe (*Figure 5*)
- Push water through filter
 - Please do not filter water back into container



5. Repeat, filtering as much water as possible
 - Unscrew filter and put in a clean place (e.g., the filter's wrapper)
 - Draw up an additional 50 mL water
 - Push water through filter
 - Be careful not to break the filter (*Figure 5*)
 - If water becomes very difficult to press through the filter it is likely clogged. Please stop filtering at this point
 - Write the volume of water pressed through the filter on the last line of the WhirlPak bag

6. Dry filter, two times
 - Unscrew syringe
 - Draw up ~50mL of air
 - Reattach syringe to filter
 - Push air through filter to remove excess water
 - Repeat
 - Remove large (60mL) syringe and discard
 - Tap the filter outlet on a clean paper towel to dry it

7. Seal filter *outlet* (*Figure 6, #1*)
 - Take one of the red rubber syringe stopper, and press it over the filter's outlet

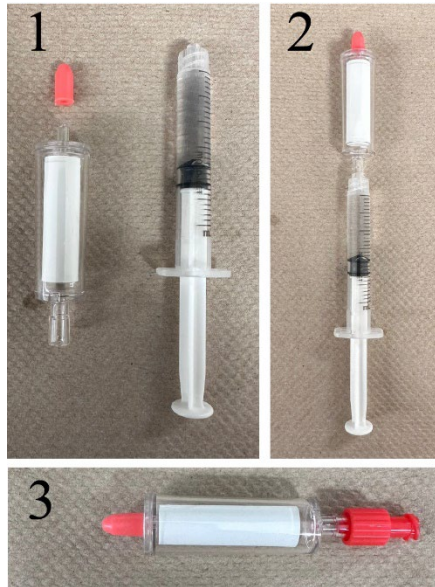


Figure 6. How to fill and seal the filter housing using the red rubber syringe stopper, 3mL syringe, and empty red cap

8. Add filter preservative (*Figure 6, #2*)
 - Unscrew the luer lock cap from the small (3 mL), pre-filled syringe and secure the syringe to the filter
 - Push the liquid from the small syringe gently into the filter housing with the sealed filter tip facing the ground
 - The liquid should *just* fill the filter case

9. Fully seal the filter (*Figure 6, #3*)
 - Remove the now empty 3 mL syringe and dispose of it
 - Acquire one of the red luer lock caps and screw its larger end over the remaining filter opening

10. Place filled, sealed, filter in labelled WhirlPak bag
 - Roll up end of bag and twist twist-ties at ends to seal the bag

11. Move on to the next tank or container on the list

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