

# Analytical and methodological development for improved surveillance of the Great Crested Newt

WC1067

## Appendix 5. Technical advice note for field and laboratory sampling of great crested newt (*Triturus cristatus*) environmental DNA



**This report should be cited as:**

Biggs J, Ewald N, Valentini A, Gaboriaud C, Griffiths RA, Foster J, Wilkinson J, Arnett A, Williams P and Dunn F 2014. Analytical and methodological development for improved surveillance of the Great Crested Newt. Appendix 5. Technical advice note for field and laboratory sampling of great crested newt (*Triturus cristatus*) environmental DNA. Freshwater Habitats Trust, Oxford.

**Version Control**

Document date: 30 September 2014  
Version Number: 1.1

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## 1. Scope of document

Environmental DNA (eDNA) is nuclear or mitochondrial DNA that is released from an organism into the environment. Sources of eDNA include secreted faeces, mucous, and gametes; shed skin and hair; and carcasses. In aquatic environments, eDNA is diluted and distributed in the water where it persists for 7–21 days, according to the detection limits of qPCR approaches and associated fragment sizes, and depending on environmental conditions (Biggs et al. 2014). Recent research has shown that the DNA from a range of aquatic organisms can be detected in water samples at very low concentrations using qPCR (quantitative Polymerase Chain Reaction) methods.

This document provides technical advice for laboratories and field staff collecting and analysing samples for qPCR analysis of great crested newt (*Triturus cristatus*) environmental DNA. The document:

- Sets out the standards required
- Sets out field and laboratory approaches for screening the presence/absence of the great crested newts
- Is designed to deliver a consistent approach, and hence comparable data, between laboratories for use in decision making.

Deviations from this protocol will need to demonstrate equivalence.

This document is based mainly on research undertaken during Defra project WC1067 “Analytical and methodological development for improved surveillance of the great crested newt” (Biggs et al. 2014). We advise that this report is used as a reference document for those carrying out great crested newt surveys using the methods described here.

## 2. Quality assurance and quality control

The methods described in this technical advice note are designed to reduce as far as possible the risk of field or laboratory generated false positive and false negative results. Quality control measures must be extended to sample collection, preservation and handling, as well as laboratory protocols, since assurances of sample quality will prove critically important to the avoidance of false negatives.

The field of aquatic eDNA is developing rapidly and it is likely that, as methods evolve, appropriate updates will need to be made to the processes detailed in this technical advice note.

### 2.1 Laboratory standard

Laboratories undertaking eDNA analysis should be able to demonstrate adequate quality assurance standards. Typically these will comprise a documented quality management system which would usually follow, or be equivalent to, the outline of ISO/IEC 17025 standard.

Ultimately it may be necessary to develop a proficiency testing scheme for eDNA analysis to enable the identification of laboratories certified as achieving the appropriate level of proficiency with the eDNA methods. At present a proficiency testing scheme for eDNA is not available because an appropriate proficiency testing methodology has not been established. Further research and development work will be needed to establish such a scheme.

In the meantime, agencies and organisations may wish to include samples from ponds known to support great crested newt and samples from sites known to be free from great crested newts to validate sampling programmes.

## 2.2 Sample acceptance

The laboratory analysing eDNA water samples should have a standard and documented sample acceptance procedure in place. This should include:

- Date and time of sample receipt
- Sample condition (i.e. has the sample container been damaged in any way)
- A visual verification of the sample volume (to detect any leakages)
- A note to confirm appropriate handling in transit (e.g. courier packaging intact).

The receiving laboratory should transfer the sample number to the sample acceptance record at this point.

## 2.3 Stability of field sampling kits

The stability of field sampling kits should be assessed through the use of an appropriate artificial DNA marker to check for unexpected decay of DNA between sampling and sample analysis. Details of the marker used, expected rates of decay and actual decay rates should be published alongside eDNA results for the target species. The marker can be chosen by the laboratory or the marker used in WC1067 can be purchased from Spygen.

## 2.4 Outcome required

Biggs et al. (2014) achieved a Limit of Quantification of  $3 * 10^{-3}$  ng/L: at present there is no evidence that great crested newt eDNA can be quantified with precision and accuracy below this level. Failure to achieve detection at this limit will lead to increased risk of false negative results for sites where great crested newt occur at low density. There should be no detection of closely related species. In the case of the great crested newt in the UK, the risk is mainly of detecting the Italian crested newt (*Triturus carnifex*) which is present at a few of locations. The primers and probe were also tested on tissue samples of marbled newt (*Triturus marmoratus*). None of these samples were amplified, confirming the suitability of the primer pair and probe for the great crested newt. The primers and probe also did not bind with the DNA of other UK native newts (smooth and palmate newt) which are in the genus *Lissotriton*.

## 2.5 Identification of risks of false positives and false negatives

There are risks of both false positives and false negatives in eDNA analysis (Darling and Mahon, 2011). Errors can occur in both field and laboratory stages of the work. Given the test's sensitivity it appears that the main risk from contamination will be from false positives.

The main risks, and their mitigation for great crested newt eDNA work, are:

- (i) Molecular assay design: mitigated in research and development phase of primer and probe design. Salt free primers should be used. The quality of the primer and of the PCR mix is assured by the standards.
- (ii) Laboratory quality control: mitigated by laboratory design and process control.
- (iii) Sampling design: mitigated by site selection procedures in field monitoring programmes.
- (iv) Uncertainty in the relationship between presence of target DNA and presence of viable target organisms: mitigated partially by research so far undertaken, and by future research increasing knowledge of great crested newt eDNA.

Table 1 summarises information on situations which may have an increased risk of generating false negatives and false positives, and potential ways to mitigate these risks. For the field sampling protocol, the risk of contamination may be greater for specialist contractors undertaking large numbers of great crested newt surveys compared to volunteers making infrequent visits to a small number of sites.

**Table 1. Risk, and mitigation, of false positives and false negatives**

Risk factor	Mitigation
<b>Field-based false positives</b>	
Cross contamination between sites (due to equipment, clothing etc.).	Ensure that there is no contact between contaminated material and the water being preserved in the sampling process.
Inflows, bringing eDNA from sites with newts into unoccupied ponds. Note that there is so far little evidence that this is a significant problem but it is a theoretical possibility.	This risk cannot be eliminated at present and its extent is not understood. Where ponds have inflows, survey teams will have to make judgements about the likely impact of any inflow. However, the majority of great crested newt ponds lack substantial inflows. The presence/absence of inflows, and whether they are wet or dry at the time of survey should be recorded in field notes.
Aquatic animals (e.g. herons, water voles) transferring newt DNA between sites (e.g. in faeces, in water trapped in fur)	This risk cannot be eliminated and the extent to which it occurs is currently unknown. Further research will be required to assess whether this is a significant risk, although at present it seems likely to be small.
<b>Field-based false negatives</b>	
Low numbers of newts	This risk is minimised by following good field protocol. Note that at present the minimum number of newts that can be detected in different waterbodies is not known. However, ponds with torch counts of zero animals in the breeding season, where newts were known to be present, have provided positive eDNA results in the breeding season.
Very wide, shallow drawdown zones may increase the likelihood of collecting water samples in areas where there has been no newt activity even though the pond is currently occupied.	To access deeper water areas it is possible that the water sampler could be added to a long pole. It is important not to enter the water as sediments will be disturbed which may contain historical great crested newt DNA. Further research data on sediment DNA is likely to be available within 6-12 months to refine understanding of this issue. In all water depths it is necessary to gently stir the water throughout its depth, without disturbing sediments, as eDNA is believed to sink. It is advisable to avoid sampling very shallow water (less than 5-10 cm deep) as it may be difficult to avoid stirring up sediment in these areas.
There is evidence that DNA is less likely to be detected in water taken from densely packed mats of vegetation; either because of a lack of newt activity or because of the difficulty of sample collection in these areas.	Avoid sampling in these areas: sample from water in areas where vegetation is suitable for egg-laying and open water areas suitable for displaying.
There is evidence that eDNA is less likely to be detected if the whole pond perimeter is not sampled.	Every effort should be made to access 20 sites around the pond for sampling. Sites where 80-90% of pond margins were accessed achieved 99.3% detection rates. Attaching the sampling ladle to an extension pole may be an option for reaching a wider range of areas. Effective cleaning of the extension pole between sites is essential. The pole must be kept separate from any equipment that is in contact with newts.

**Table 1 (cont). Risk, and mitigation, of false positives and false negatives**

Risk factor	Mitigation
<b>Laboratory false positives</b>	
Contamination of eDNA sampling kits.	Mitigation is largely ensured by good laboratory design, set-up and processes, particularly separation of the sample preparation room from all other stages of the process.
Contamination during DNA amplification.	Mitigation is largely ensured by physical separation of the different stages of the PCR process, use of dedicated equipment and lab coats for each stage and a uni-directional work flow from clean to DNA contaminated rooms.
The risk of contamination in the laboratory is likely to be greatest when larger numbers of samples (>20) and multiple batches of samples are handled.	Mitigation is largely ensured by good laboratory design, set-up and processes. It is to be expected that handing of smaller batches of samples (i.e. <20 samples), in single trials, will be easier than larger throughput operations.
<b>Laboratory false negatives</b>	
Very low eDNA concentrations.	Samples with DNA amounts below the Limit of Detection will generate false negatives. It is not currently possible to mitigate this risk.

## 2.6 Laboratory specifications

### 2.6.1 How the laboratory should be set up

The set-up of an eDNA laboratory should broadly follow the outline below. Note that this is not a detailed specification for building a laboratory: rather it provides guidance on the standard which is needed.

Successful eDNA work has so far been undertaken both in laboratories designed to standards established over the last 20 years for ancient DNA (aDNA) work (Knapp et al., 2012), and in more conventional DNA labs. There is as yet no evidence available to evaluate whether these different set-ups produce different results.

The main principles of the laboratory set-up should be (PHE, 2013):

- **Physical separation of pre and post-PCR work:** to prevent amplified DNA from contaminating samples there should be physical separation of pre and post PCR stages of the work. This should include separation of the area where sampling kits are prepared.
- **Unidirectional workflow:** The arrangement of activities in the rooms should be unidirectional to reduce potential for contamination. This can be achieved by physically having one room leading to another or by set working practices.

Two potential layouts of facilities based on existing constructed systems are exemplified below (Figure 1). The simpler design has some recognised limitations which are noted in the figure. Good results are known to have been produced in higher specification laboratories. The main features of the designs are:

- **Reagent preparation clean room:** a clean DNA free room is needed for the preparation of field sampling kits. Samples containing DNA should never be brought into this room and no DNA extractions or PCRs are performed in this room<sup>1</sup>.
- **Nucleic acid extraction room:** the only area where DNA extractions are performed, and an area where PCR products and stocks of cloned material have not been handled.
- **Amplification room:** this is the area where PCR machines are housed.

The schematic designs shown in Figure 1 fulfil these criteria.

### 2.6.2 Appropriate precautions to avoid laboratory contamination

As PCR products are ubiquitous in post-PCR laboratories it is important to make sure that no consumables or equipment for the DNA facility have been sourced from laboratories which undertake post-PCR amplification analysis.

Full body suits have been adopted by some eDNA laboratories for work in the sample kit preparation room and the eDNA sample preparation room, including full body suit, face masks, face shields and hairnets. In other rooms disposable laboratory coats are sufficient. Dedicated clean room shoes are useful to reduce carry-over contamination. Wearing two pairs of gloves will prevent exposure of skin when changing gloves. However, not all laboratory managers consider 'suited-up' necessary, preferring separation of staff as the contamination control method (i.e. staff do not move between pre- and post-PCR laboratories). Those working with full body suits regard this approach as good practice for rare DNA work which generally reduces the amount of DNA present in the rooms to very low levels. Face masks reduce the breathing out of DNA which has been inhaled outside the clean rooms.

To reduce the risk of DNA contamination regular bleaching of the laboratory should be undertaken weekly. qPCR work should be undertaken inside a cabinet with UV light and in a room which is also lit by UV light outside the cabinet; to control aerosol DNA. Although UV lights are widely recommended for decontamination they need to be high power and close enough to the surface for decontamination to be effective and only then for low level contamination - cleaning and liquid decontamination is more effective (for detailed discussion see Champlot et al., 2010). They are also used in some laboratories to keep levels of environmental DNA low, including UV irradiating the facility when it is not in use.

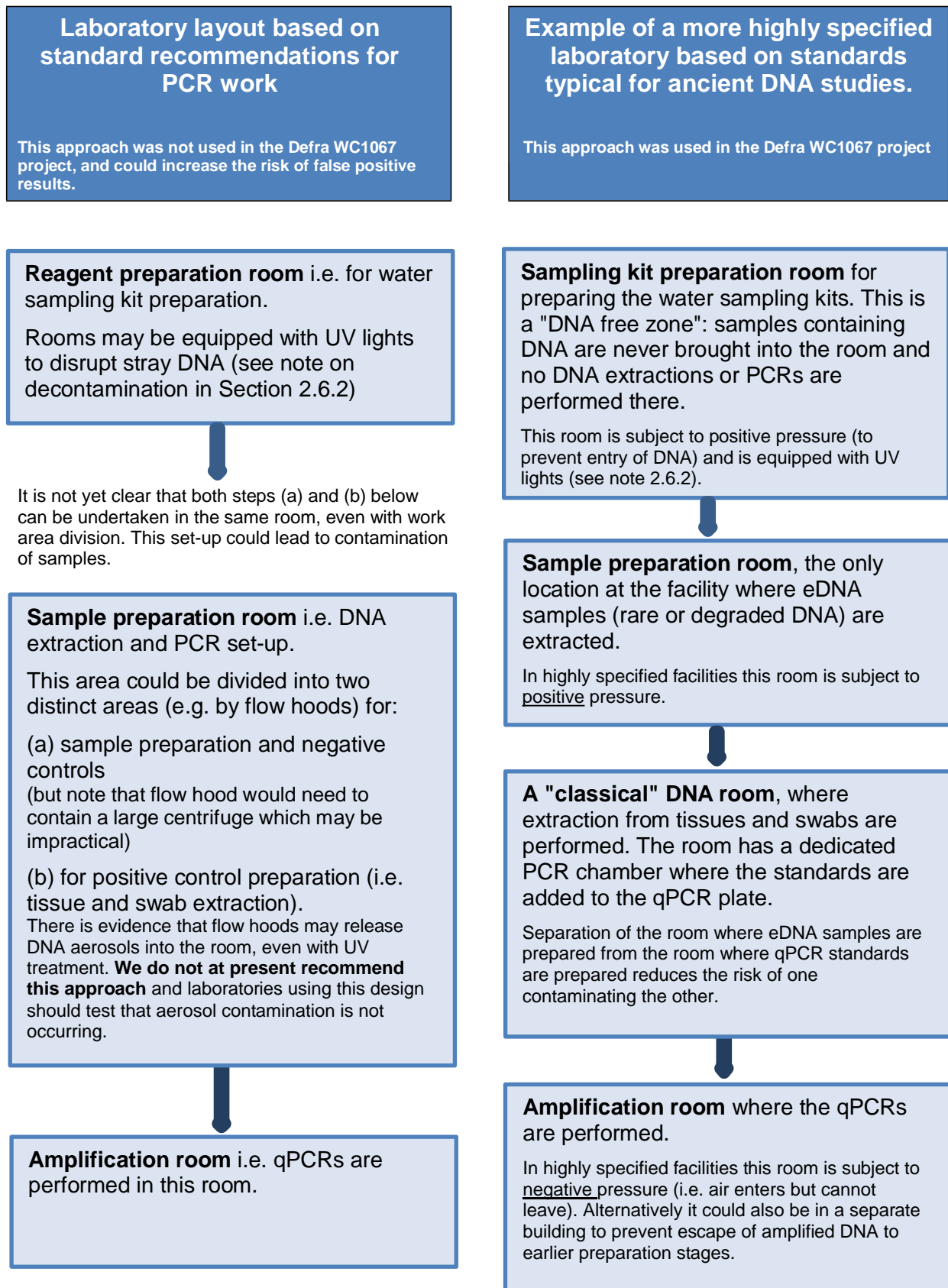
Dedicated laminar flow hoods and fume hoods for DNA extraction and manipulation can reduce the risk of contamination still further. However, note that laminar flow hoods and fume hoods can under some circumstances make contamination worse by circulating contaminating aerosols around the laboratory. Most PCR hoods either do not have air flow, or are used switched off, providing a dedicated work station that is contained and can be easily decontaminated.

Further useful features are a positive pressure system and HEPA-filtered air conditioning. Some teams regard positive and negative pressure as desirable features to control contamination effectively. However, others suggest that procedural aspects are more important. At present it is not possible to tell which of these positions is correct. The more stringent standards of ancient DNA workers normally include positive / negative pressure and several successful laboratories working with eDNA have used this set-up. However, other groups have produced published results (e.g. Pilliod et al., 2013) without such systems. A highly specified laboratory (e.g. a forensic laboratory) may also have dedicated staff for each area because people are a major source of contamination. Vestibules with shoe/coat changing are effective techniques to prevent transfer of DNA in a highly specified laboratory, but can be adopted less expensively in less well specified laboratories by having dedicated shoes and coats for each laboratory.

<sup>1</sup> It is possible that a Class II cabinet in a non-DNA free room could be used for this step. If this approach is used it would be advisable for laboratories to demonstrate that this process did not lead to contamination of sample test kits. Cabinets are at risk of contamination from DNA aerosols which can be present in DNA laboratories even with UV lighting.



**Figure 1. Examples of laboratory specifications proposed or in use for eDNA work.**



### 3. Field protocol

Field sampling should be undertaken by a suitably trained and experienced great crested newt surveyor (trained volunteer or professional). At present it is believed that eDNA water sampling does not disturb newts enough to justify the procedure being licensed by the national regulatory authority.

A single visit to the target pond should be made between mid-April and June, during the newt breeding season. eDNA samples can be collected at any time of day and in any reasonable weather conditions, including light rain. It may be best to avoid heavy rain as this makes sampling more difficult and might increase the risk of cross contamination (e.g. splashing of mud which could contain great crested newt DNA from wet ground). There is evidence that unpreserved amphibian eDNA decays slightly more quickly in full sun than shaded conditions, becoming undetectable after 8 and 11 days respectively (Pilliod et al., 2014), but as long as samples are preserved the impact on detection should be slight.

#### 3.1 Sampling equipment

The field sampling equipment used by Biggs et al. (2014) has five components (Figure 2):

- A sterile 30 mL ladle
- A sterile self-supporting Whirl-Pak plastic bag with 1 L capacity
- A sterile 10 mL pipette to resample the pond water
- Six sterile 50 mL centrifuge tubes containing preservative (Absolute Ethanol (200 Proof), Molecular Biology Grade, Fisher BioReagents (Product Code: 10644795), sodium acetate and other markers)
- Two pairs of sterile gloves.

**Figure 2 Sampling equipment used for eDNA water samples by Biggs et al. (2014)**



Kits can be stored at room temperature before use in an appropriate solvent store, consistent with Home Office regulations, and should be used within about two weeks of receipt. The time between kit receipt and use should be noted (see Section 5.1). Use one kit per pond up to an area of 1 ha. Beyond this, use an additional kit per hectare. However, note that as yet there is no practical experience of the effectiveness of kits used on ponds greater than 1 ha in area. Note that sampling techniques are still developing rapidly in this field and alternative preservatives to ethanol are currently being sought.

### **3.2 Field water sample collection protocol**

The field sampling protocol should follow the steps outlined below. Gloves should be worn at all times during the sampling process, replacing the gloves between sample collection from the pond and pipetting into the sterile sub-sample tubes. Samples should be collected without entering the water, i.e. the surveyor stands only on the pond bank or muddy pond edges. This prevents disturbance of the substrate and may limit cross-contamination.

#### **Stages of field sampling protocol**

- Step 1 Identify where 20 samples will be taken from the pond. The location of sub-samples should be spaced as evenly as possible around the pond margin, and if possible targeted to areas where there is vegetation which may be being used as egg laying substrate and open water areas which newts may be using for displaying.
  - Step 2 Open the sterile Whirl-Pak bag by tearing off the clear plastic strip c 1cm from the top (along the perforated line), then pulling the tabs. The bag will stand-up by itself.
  - Step 3 Collect 20 samples of 30 mL of pond water from around the pond (see 1 above) using the ladle (fill the ladle), and empty each sample into the Whirl-Pak bag. At the end the Whirl-Pak bag should be just under half full (600 mL).
- NOTE: Before each ladle sample is taken, the pond water column should be mixed by gently using the ladle to stir the water from the surface to close to the pond bottom without disturbing the sediment on the bed of the pond. It is advisable not to sample very shallow water (less than 5-10 cm deep).
- Step 4 Once 20 samples have been taken, close the bag securely using the top tabs and shake the Whirl-Pak bag for 10 seconds. This mixes any DNA across the whole water sample.
  - Step 5 Put on a new pair of gloves to keep the next stage as uncontaminated as possible.
  - Step 6 Using the clear plastic pipette provided take c15 mL of water from the Whirl-Pak bag and pipette into a sterile tube containing 35 mL of ethanol to preserve the eDNA sample (i.e. fill tube to the 50 mL mark). Close the tube ensuring the cap is tight.
  - Step 7 Shake the tube vigorously for 10 seconds to mix the sample and preservative. This is essential to prevent DNA degradation. Repeat for each of the 6 conical tubes in the kit. Before taking each sample, stir the water in the bag to homogenize the sample - this is because the DNA will constantly sink to the bottom.
  - Step 8 Empty the remaining water from the Whirl-Pak bag back into the pond.
  - Step 9 The box of preserved sub-samples is then returned at ambient temperature immediately for analysis. If batches of samples are collected and stored prior to analysis they should be refrigerated at 2-4° C. Kits can be stored for up to one month in a refrigerator before analysis. It is not necessary to freeze samples. Freezing may damage storage bottles, which can lead to leaking during transit, and also unnecessarily increases costs by requiring refrigerated transport. The length of time eDNA samples are stored in a refrigerator prior to analysis should be recorded and passed on to the analysing laboratory. Use an appropriate labelling system to ensure that the kits are supplied with a unique reference number.

## 4. Laboratory protocol

### 4.1 Introduction

This section describes the laboratory protocol for analysis of eDNA samples. It is assumed that laboratory staff are familiar with the techniques for using the proprietary products specified.

It is important that the analysing laboratory has no prior knowledge of whether sites being tested do or do not have newts. Samples should be identified only by a unique reference number which contains no site identifying information.

### 4.2 Analytical methods

#### Primers and probes

Great crested newt (*Triturus cristatus*) DNA should be amplified using the primers and probes listed in Appendix 2. They amplify a fragment of the mitochondrial cytochrome oxidase I gene (cytb). It may be desirable for laboratories undertaking analyses to demonstrate that these primers and probes have been tested *in vitro* against real great crested newt tissue (which can be collected by external swabbing), and *in situ* from real ponds with great crested newts (unless they have already undertaken eDNA work with great crested newts). There are a number of amphibian biologists around the UK who have licenses to swab newts and they can be contracted to do this work. An alternative approach to standardisation is to purchase synthetic DNA.

#### Water

Water used in eDNA analysis should be ultrapure water for molecular biology grade, which can either be purchased or made in the laboratory, using proprietary equipment.

#### 1. DNA extraction

DNA should be extracted using the DNA Blood and Tissue kit (Qiagen®) following the manufacturer's instructions.

- Step 1** For each sample from a site, the six subsamples per site should be centrifuged at 14000 x g<sup>1</sup>, for 30 minutes, at 6 °C and the supernatant discarded.
- Step 2** 360 µL of ATL Buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen®) is added to the first tube, the tube is vortexed for several minutes (time depends on degree of film accumulation on tubes) and the supernatant poured into the second tube. This operation is repeated for all the six tubes, resulting in the 6<sup>th</sup> tube containing the ATL buffer that has been vortexed sequentially in each of the six sample tubes. Vortexing is needed to remove films of DNA which become attached to the tubes at high centrifuge speeds. Flicking the tube or pipetting have not been found sufficiently vigorous to remove these films. Other kits may be suitable for this step but would need to be evaluated, perhaps as part of a proficiency testing process.
- Step 3** The supernatant in the sixth tube, containing the DNA concentrated from all 6 subsamples, is transferred to a 2 mL tube and the DNA extraction performed following the manufacturer's instructions. The DNA extraction should be performed in the room or laboratory area dedicated for degraded DNA samples.
- Step 4** An extraction control should be performed at the same time to monitor possible

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<sup>1</sup>The centrifugation speed suggested originally by Ficetola et al. (2008) was 5500 x g. Internal tests made by Spygen indicated that better results were found with the highest centrifugation speed, which led to the adoption of 14,000 x g for the Great Crested Newt DNA extraction.

laboratory contamination. The extraction control is undertaken using an 11<sup>th</sup> tube containing buffers alone and no sample (i.e. no alcohol mix and no pond water). Note that the quality of the alcohol (i.e. absence of DNA contamination) is assessed with the negative controls in the field. These can be either out of range sites where great crested newts are definitely absent or sites within the newt's range where there is high certainty that newts are absent. If no negative field sites are available in a study, a different approach may be needed. In the analytical process the extraction control sample is, from Step 4 onwards, processed as a normal sample.

Additional control samples may be added to the process depending on where it is believed contamination may be originating.

## 2. qPCR

Step 5 DNA inhibition should be tested by adding a known amount of an artificial gene to the sample and running qPCR in duplicate. If a different than expected  $C_t^2$  value is observed in a least one replicate, the sample should be considered inhibited. In this instance dilute the sample twice before amplification with great crested newt primer and probes.

Step 6 qPCR analysis. Each sample should be run in 12 replicates. A dilution series of *T. cristatus* DNA, ranging from  $10^{-1}$  ng  $\mu\text{L}^{-1}$  to  $10^{-4}$  ng  $\mu\text{L}^{-1}$  (increments  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ) and measured using a Nanodrop ND-1000 or equivalent, should be used as a qPCR standard. The qPCR standards are made using DNA extracted from great crested newt tissue samples, and the quantification made on extracted DNA before the dilution. Samples should be run on a BIO-RAD® CFX96 Touch real time PCR detection system or equivalent.

Note that the standards are the positive controls for qPCR in this approach (i.e. assuring that the method successfully detects DNA when present). Negative controls are provided by one extraction blank, which is run with 12 replicates, as a normal sample, and with four qPCR negative controls which also run during the qPCR step, using ultrapure water for molecular biology grade.

Step 7 The quantitative PCR is performed in a final volume of 25  $\mu\text{L}$  made up from:

- 3  $\mu\text{L}$  of template DNA
- 12.5  $\mu\text{L}$  of TaqMan® Environmental Master Mix 2.0 (Life Technologies ®)
- 6.5  $\mu\text{L}$  of ddH<sub>2</sub>O
- 2  $\mu\text{L}$  of primer (1  $\mu\text{L}$  each of primer 10  $\mu\text{M}$  TCCBL and TCCBR)
- 1  $\mu\text{L}$  of probe (2.5  $\mu\text{M}$  TCCB Probe)

Step 8 The PCR is performed under thermal cycling at 56.3 °C for 5 minutes and 95 °C for 10 minutes, followed by 55 cycles of 95 °C for 30 seconds and 52°C for one minute.

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<sup>2</sup>( $C_t = C_t$  threshold value, the number of PCR cycles after which amplification becomes exponential)

## 5. Data recording and reporting

Accurate detailed records of the sites surveyed should be kept by the commissioning ecologists for reporting, reference and auditing purposes.

### 5.1 Sampling information

Sampling kits should be identified by a unique identifying code when provided to field ecologists. All site information should be associated with this unique number. Laboratory staff do not need further site reference information.

The commissioning ecologists should maintain records which include:

- Site name
- Nearest settlement (provides double check against grid reference errors)
- County (provides double check against grid reference errors)
- Time between receipt of sampling kit and date of sampling
- Date of sampling
- Personnel collecting sample
- Ordnance Survey grid reference, ideally to 1 m (i.e. a 12 figure grid reference)
- Site maps showing locations of sites
- Percentage of pond perimeter that is accessible for survey
- Data on inflows, and whether these were wet or dry at the time of survey
- If available, data on presence and number of great crested newt recorded during eDNA collection to help with further assessment / refinement of this technique
- Information on any difficulties experienced during sample collection.

### 5.2 Laboratory data

The laboratory should maintain records which include:

- Personnel involved identified
- Date of kit preparation
- Duration of storage of samples once returned from the field
- Dates of analysis
- Details on type and any degradation of the marker DNA in sample kits
- A record of any modifications to standard operation procedures of laboratory equipment.

Standard laboratory data should be maintained by the laboratory.

Information on sample inhibition should be reported with the reporting of positive or negative DNA results.

At present there is no intention to archive eDNA samples although this may become necessary in the future.

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## Appendix 1. Frequently asked questions about eDNA sampling from volunteer and non-specialist surveyors

### **What is eDNA?**

eDNA in the case of larger organisms, is DNA that is collected from the environment in which an organism lives, rather than directly from the plants or animals themselves. In aquatic environments, animals including amphibians and fish, shed cellular material into the water via their saliva, urine, faeces, skin cells etc. This DNA may persist for several weeks, and can be collected through a water sample, and analysed to determine if target species of interest have been present in the waterbody.

### **Why must surveyors remain out of the pond?**

There is a considerable risk of contaminating your pond sample by bringing in Great Crested Newt DNA in mud and water from other areas on your boots and equipment. This is a real risk: DNA can remain on surfaces even after they have been dried, and can persist in soil for many years. There are recorded examples of eDNA cross-contaminating pond water samples from surveyor's boots.

### **Why are sampling points spread around the pond?**

Existing data shows that eDNA can be very patchy depending on where the animals have been. By sampling in many areas you considerably increase your chance of collecting their DNA successfully.

### **Why is the water column mixed before sampling?**

DNA 'sinks' and so will often be present in larger amounts close to the pond bottom. However, it is important not to collect sediment because it is currently thought that DNA may persist in the sediment for substantially longer than in the water column. If you collect sediment, there is a risk your sample might show a false positive indicating great crested newts were present recently, when in fact this was a long time in the past.

### **Why is such a large volume of water collected?**

In this methodology we collect a larger volume of water than previous methods have recommended (e.g. Thomsen et al. 2012). Our experience indicates that collecting a larger volume of water than was taken by Thomsen et al. (2012) is important to the success of the method.

### **Does it matter if I get things like duckweed, algae or zooplankton in my sample?**

No, small amounts don't matter. However try not to collect bottom sediment in the sample, because the DNA can be absorbed by sediment and may give false positive results (see above).

### **What happens if I spill the preservative - or the sample tube itself**

If you spill some of the preservative from one of the tubes, just add proportionally less water from your pond sample. The samples from all six tubes are later combined for the laboratory analysis, so it's not disastrous if some sample is lost.

### **Will samples degrade in the post?**

The preservative (alcohol) in the sample bottle will slow, but not eliminate, degradation of any DNA. Keeping the samples refrigerated also slows this process. The rate of decay during posting at ambient temperatures will be faster, but it will not be sufficient to degrade the sample completely.

### **What evidence is there to support the use of this technique?**

Defra project WC1067 has demonstrated the effectiveness of environmental DNA in the detection of Great Crested Newts. In detailed field studies eDNA detected Great Crested Newts 99.3% of the time in ponds where they were known to occur. When used by volunteers surveyors, eDNA detected Great Crested Newts at 91% of ponds where they were known to be present. No false positives were recorded from sites either outside or within the known range of the newt.



## Appendix 2 Details of primers and probes

Primers are salt free and HPLC-purified.

<b>Primer</b>	<b>Sequence (5'-3')</b>	<b>Fragment</b>	<b>Gene</b>
TCCBL	CGTAAACTACGGCTGACTAGTACGAA	81	Cyt-b
TCCBR	CCGATGTGTATGTAGATGCAAACA	81	Cyt-b
<b>Probe</b>			
TCCB	CATCCACGCTAACGGAGCCTCGC	81	Cyt-b

### Degradation control

A length of artificial DNA is added to the samples to assess post-sampling degradation. This DNA does not have an analogue in the natural world so it can clearly be separated from all DNAs that can be sampled in the field. The structure of the molecule is commercially confidential to Spygen so is not reproduced in this guide. Laboratories may either design their own synthetic DNA or purchase material from Spygen.

## **Acknowledgements**

We would like to thank all those who helped with Defra project WC1067 including the landowners who facilitated access to their sites, and particularly the many people and groups who volunteered time and resources to collect eDNA samples. This includes NARRS and PondNet volunteers, the team in Wales co-ordinated by Matt Ellis of Natural Resources Wales, who generated an excellent dataset for the detailed methodological component of the project, and Tom Langton who not only collected many eDNA samples, but provided access to a dataset from Suffolk extending over 20 years which has helped us to better interpret the relationship between eDNA and Great Crested Newt abundance. The project lead was Natasha Chick (Defra) and the Steering Group was Matt Ashton (Defra), Pete Brotherton (Natural England), Paul Edgar (Natural England), John McKinneil (Scottish Natural Heritage), Katharine Woods (Natural England) and Anna Robinson (JNCC). Our thanks also to Barbara Zweifel for delivering eDNA samples to France for analysis.

Specific thanks for technical advice on the preparation of this document are given to Neil Boonham (FERA), Simon Creer (Bangor University), Helen Rees (ADAS) and Kerry Walsh (Environment Agency), and to Mike Wilkinson and Katharine Woods (both of Natural England).