Performance of the Freedom4 for detection of *Batrachochytrium dendrobatidis*

ubiquitome

Introduction

The world's amphibians are under threat with many species either on the verge of disappearing or already extinct. Recent statistics show that 30% of species are critically endangered with 165 species already thought to have been lost (http://www.amphibianark.org/the-crisis/frightening-statistics/). Though climate change and habitat destruction are involved, the mechanism leading to extinction is the spread of amphibian pathogens.

The first of these destructive pathogens to be described was the Chytrid fungus *Batrachochytrium dendrobatidis* or Bd. This is a parasitic fungus that lives on the skin of frogs (Longcore et al, 1999). Infection leads to the disease chytridiomycosis that causes the skin to thicken through hyperkeratosis (Brutyn, 2012). Amphibians absorb water and electrolytes (and in some salamanders, air) directly through their skin. Hyperkeratosis prevents this, resulting in electrolyte imbalances that in frogs causes the heart to stop.

Other pathogens also contribute to population decline. Recently a related fungus *Batrachochytrium salamandrivorans* (Bs) has raised fears for salamanders (Stokstad, 2014). And it is not only fungi. Outbreaks of Ranavirus (RV), a double-stranded DNA virus from the Iridoviridae family, (Chinchar, 2002) is having a serious impact.

Dr Robert Puschendorf (Plymouth University, UK), in collaboration with Prof. Janzen and Dr Hallwachs (University of Pennsylvania, USA) has been studying amphibian populations in the Área de Conservación Guanacaste, Costa Rica (www.acguanacaste.ac.cr). They aim "to understand the fine scale distribution, diversity and prevalence patterns of [Bd and RV] in a tropical system." To do this, they use qPCR to diagnose Bd or RV from samples collected from the field. Captured frogs are swabbed and then released. The swabs are then sent to a laboratory for processing. This creates a considerable delay between frog capture and diagnosis. If diagnosis could be performed in the field, real-time relationships between infected frogs and the environment could be observed more efficiently. In addition, near real-time detection of infection in an at-risk population would make it possible to rapidly implement a species rescue plan. This could make the difference between saving and losing a population.

The Freedom4 offers a platform to perform qPCR in the field. Dr Puschendorf is interested to use the Freedom4 for in-field diagnosis of amphibian pathogens. The first step is to show that Freedom4 can perform diagnostic assays for Bd and RV.

Materials and Methods

qPCR instruments

All reactions were run in parallel on the Freedom4 (Ubiquitome, New Zealand) and the LightCycler 480 (Roche, Switzerland)

Template

Sample of Bd and RV DNA were obtained from Dr Puschendorf (Plymouth University, UK). On arrival, the concentration of each sample was measured using the Qubit fluorometer (Invitrogen, USA) and the High Sensitivity Double Stranded DNA detection kit (Invitrogen, USA). The RV sample had a concentration of 100pg/µl and the Bd sample had a concentration of 15ng/µl.

qPCR amplification chemistry and assays

All work reported here used the Light Cycler 480 2x SYBR Green Master Mix I system from Roche

(Roche Cat No. 04 707 516 001). qPCR assays for Bd and RV are as described in Boyle et al (2004) and Jaramillo et al (2012), respectively. All oligonucleotide probes and primers were synthesised by Integrated DNA Technologies (USA).

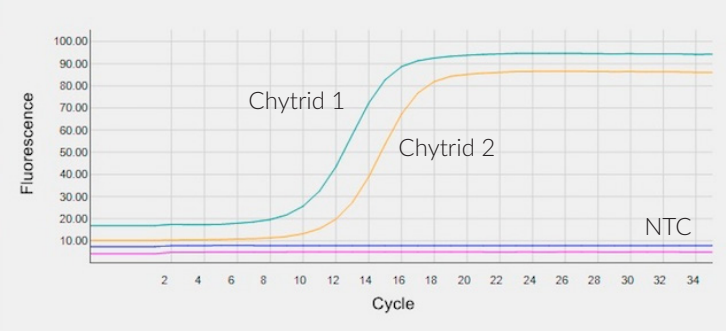
Reaction mixes consisted of 10µl SYBR 2x Green Master Mix I, 1µl of Forward and Reserve primer (or 2µl of IDT primer mix) and 1 to 3µl of DNA template. PCR grade water was added to a final volume of 20µl.

Results and Discussion

Figure 1 shows that the Bd qPCR assay gives the same amplification output as the LightCycler 480 in side-by-side runs. Ranavirus was used as a cross-reaction control. No amplification was observed when the Bd qPCR assay was used with the Ranavirus template. Similar results were obtained from 6 independent runs of the Freedom4 and included runs powered entirely from the Freedom4's on-board battery.

The Bd template was diluted ten-fold to give a dilution series of the template. Duplicate dilutions were run in a single run on the LightCycler 480 and two back-to-back runs on the Freedom4. The Cq of each reaction is graphed in Figure 2.

Freedom4



LightCycler 480

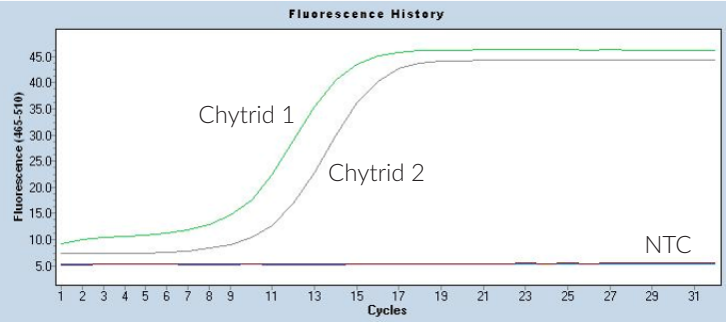
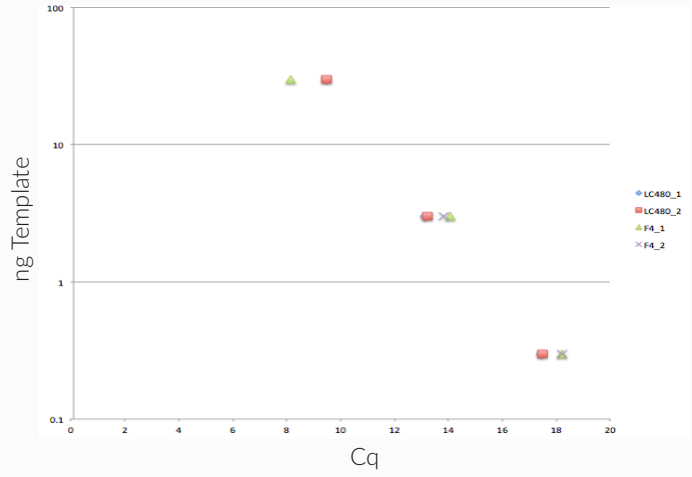


Figure 1: Bd assay used in side-by-side runs on the Freedom4 and the LightCycler 480. Chytrid 1 used 3µl of the Bd sample; Chytrid 2 used 1 µl of the Bd sample; Ranavirus used 3µl of the RV control; NTC did not contain DNA template.



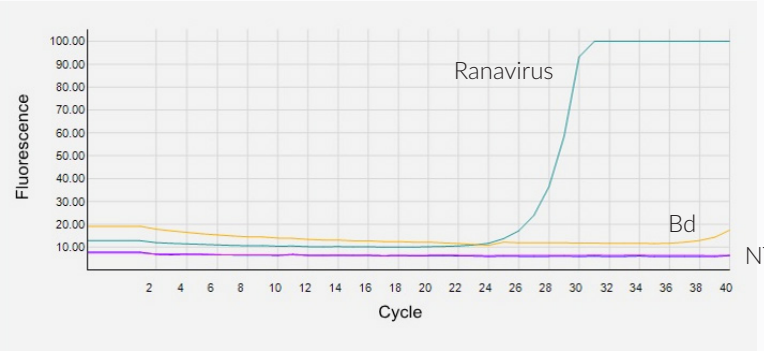
ng DNA	LC480_1	LC480_2	F4_1	F4_2
30	9.48	9.48	8.17	-
3	13.15	13.23	14.05	13.83
0.3	17.45	17.48	18.22	18.21

Figure 2: Ten-fold serial dilution of the Bd template amplified using the Bd qPCR assay. Samples were amplified in duplicate on a single run of the LightCycler 480 and in subsequent runs on the Freedom4. An artefact resulted in no Cq call for one of the Freedom4 reactions. No amplification was detected in the NTC controls.

Figure 3 shows an example of the RV qPCR assay run against the RV and Bd samples in parallel experiments performed on the LightCycler 480 and the Freedom4. This experiment was repeated and gave the same result. Some non-specific amplification was evident with the Bd template that may have arisen from the high DNA concentration used in the qPCR reaction. Non-specific amplification was also noted with the LightCycler 480 from both the No Template Control (NTC) and Bd samples indicating either primer dimer or sample cross-contamination on this instrument.

These results demonstrate that the Freedom4 can perform the diagnostic assays for both Bd and RV with similar results to the LightCycler 480.

Freedom4



LightCycler 480

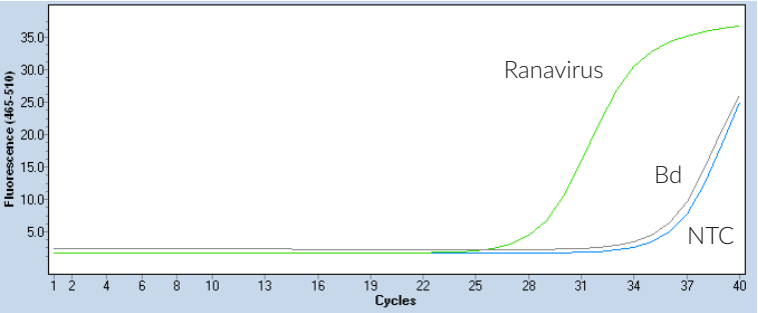


Figure 3: RV assay run side-by-side on the Freedom4 and the LightCycler 480. Ranavirus used 3µl of the RV sample; Bd used 3µl of the Bd DNA sample; NTC did not contain DNA.

References

Boyle DG, Boyle DB, Olsen V, Morgan JA, Hyatt AD. (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Organ 60(2):141-8.

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Chinchar VG. (2002) Ranaviruses. (family Iridoviridae): Emerging cold-blooded killers. Archives of Virology 147: 447–470.

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