1 **Short communication:** Development of HyBeacon® probes for the forensic detection of

2 *Panthera*, rhinoceros, and pangolin species.

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1   **Abstract**

2   The Illegal Wildlife Trade (IWT) represents a multi-billion dollar black-market industry

3   whereby wild species are illegally taken from their natural environment and sold. A common

4   question asked by wildlife forensic scientists pertains to species and/or genus identity, which

5   currently requires multi-step processing. Our work details the development of three

6   HyBeacon® probes, used for the presumptive detection of rhinoceros, pangolin and key target

7   species in the *Panthera* genus. The approach can be performed in a single tube using melt curve

8   analysis and provide rapid assessment of sample identity. Using synthetic DNA of

9   representative species, early data suggest the approach is sensitive enough to achieve species

10 identification with <10 cells. Future development and assay validation can allow the rapid

11 screening of multiple seized items before confirmatory DNA sequencing.

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13 **Key Words:** Species detection; endangered species; HyBeacon; wildlife forensics;

14 presumptive detection; rapid screening.

The Illegal Wildlife Trade (IWT) is the fourth most lucrative form of illegal trade after guns and narcotics [1]. Three of the most highly trafficked groups of species include members of the *Panthera* genus, including the tiger (*P. tigris tigris*) and Sumatran tiger (*P. tigris sondaica*), jaguar (*P. onca*), and leopard (*P. pardus*), where bone can be ground to powder for use in traditional medicines [2,3]; members of the Rhinocerotidae family, including the white (*C. simum*), black (*D. bicornis*), Indian (*R. unicornis*), Javan (*R. sondaicus*) and Sumatran (*D. sumatrensis*) rhinoceros, where the horn is ground into powder for use in traditional medicines [4]; and members of the Manidae family, including four species of Asian pangolin (*Manis spp*) and four species of African pangolin (*Phataginus spp* and *Smutsia spp*), where the meat is use in cooking and the scales are used in traditional medicines [5]. Currently, several molecular methods and techniques are used for forensic species identification, including Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) [6,7], Random amplified polymorphic DNA (RAPD) [8,9], melt curve analysis using intercalating dyes [10,11], and the gold standard, DNA sequencing [12,13]. HyBeacon probe technology offers promising results in the field of forensics and other scientific disciples including STR detection for forensic applications [14,15], SNP detection for personalised medicine [16], and species identification for food standard investigations [17]. HyBeacon probes work by emitting a fluorescent signal when hybridized to complimentary DNA sequences. The temperature at which the probes dissociates from the target is determined by the degree of complementarity between the probe and the sequence to which it is bound with data being observed as an end-point melt curve on a fluorescent Real Time PCR instrument. The probes can be labelled with different dyes allowing the multiplex detection of different DNA sequences [18].

In this study, mitochondrial Cytochrome Oxidase I (COI) sequences from rhinoceros and pangolin species, and Cytochrome b (Cytb) sequences for tiger, jaguar and leopard species were downloaded from the GenBank collection [19] together with representatives of other

26 closely related species and aligned and edited to the same length using Clustal Omega [20].

27 The sequences were examined in MEGA 6.0 [21] to identify a ~30 bp region that showed a

28 high degree of homology within each 'target' species but were heterologous to other, closely

29 related, 'non-target' species. Once identified, the sequence was ordered in as a fluorescently

30 labelled HyBeacon probe from LGC Biosearch (Table 1). Internal dt fluorophore labelling was

31 used to allow multiplexing; rhinoceros (FAM), pangolin (JOE), *Panthera* (TAMRA). Due to

32 difficulty in obtaining DNA samples from the species under study, both specificity and

33 sensitivity studies used synthetic DNA constructs following the approach taken by [11]. The

34 specificity of each probe to its target and non-target species was assessed through the use of

35 30bp long synthetic Reverse Complement (RC) oligos (Eurofins) which were the designed for

36 each species based on the downloaded GenBank sequences (Table 1). Multiplex reactions for

37 testing specificity were performed in 20 µl volumes containing a final concentration of 0.15

38 µM each HyBeacon probe, 0.3 µM of the RC oligo under assessment and 2x Phusion HF buffer

39 (ThermoFisher). Three replicate reactions were performed for each species including negative

40 controls following [22]. Melt curve analysis was performed on a Rotor-Gene Q 5plex HRM

41 System (QIAGEN) with the following melt curve setting: 95°C for five minutes, ramp from

42 95°C to 30°C, falling 1°C each step, hold at 30°C for 60 seconds and final melt from 30°C to

43 80°C rising by 1°C each step. Temperature specific Analytical Thresholds (AT) were

44 established for each species by measuring the fluorescence recorded at each species melt

45 transition in relation to the negative control. This was done by calculating the average noise

46 observed in the negative control samples at each species melt temperature plus three standard

47 deviations. This threshold allowed for the unbiased differentiation between target peaks and

48 negative controls. The melt temperatures of each species were recorded to establish if they

49 could be uniquely identified.

50   The sensitivity of the proposed assay was assessed through the asymmetric PCR amplification

51   of a synthetic DNA strand (100-150 bp long) that matched the species of interest

52   (ThermoFisher GeneArt Strings). Species-specific primers (Eurofins) were designed to

53   amplify the synthetic template DNA (Table 1). The known concentration of the synthetic DNA

54   was used to calculate copy number before undergoing serial dilutions to obtain working

55   solutions of 50,000; 5,000; 500; 500; 5 and 0.5 copies per µl. Asymmetric PCR [23] was

56   performed in 20 µl volumes containing 0.15 µM each target species probe, 0.5 µM each species

57   forward primer, 0.125 µM each species reverse primer, 200 µM dNTP, Phusion HF buffer (1x),

58   0.02 U/µl of Phusion hot start II DNA polymerase (ThermoFisher) and 2 µl synthetic template.

59   Three replicate reactions were performed at each concentration including negative controls

60   following [22]. Thermal cycling conditions were as follows: 98°C for 30 seconds, followed by

61   40 cycles of 98°C for 10 seconds, 60°C for 2 seconds, 72°C for 10 seconds. The melt was

62   performed immediately after PCR following the same settings as that reported for the

63   specificity study. The Limit of Detection (LOD) was established by performing a t-test to

64   establish when there was no longer a significant difference between blank samples and those

65   containing progressively lower amounts of input DNA.

Table 1. HyBeacon probe binding locations of target species and closely related species of rhinoceros, pangolin and *Panthera* species with the observed melt Temperature.

Scientific Name	Common Name	Oligo ID	Target DNA sequence (COI)	Observed Tm (°C)	Downloaded Sequence (n)	Species Coverage
Panthera Probe		–	5'- C C C T C A A A A A A G A C A T* T T G G C C T* C A T G G T A A G -3'	–	–	–
P. tigris tigris	Tiger	RC0	5'- C T T A C C A T G A G G C C A A A T G T* C T T T T T G A G G G -3'	59.8	100	100%
P. tigris sondaica	Sumatran tiger	RC2	5'- . . . . A . . . C . . . . A -3'	52.8	10	100%
Panthera onca	Jaguar	RC8	5'- . C . . . . A . . . C . . . . -3'	43.8	10	100%
Panthera pardus	Leopard	RC10	5'- . C . . . . A . . . C . . . . A -3'	43.8	10	100%
Rhinoceros Probe		_	5'- A T A A T T G T A G T* A A T A A A G T* T A A T G G C A C C -3'	_	_	_
Rhinoceros Reverse Complement Olgio		(RC 0)	5'- G G T G C C A T T A A C T T T A T T A C T A C A A T T A T -3'	_	_	_
Rhinoceros unicornis	Indian rhino	(RC 1)	5'- . . G . . . . . C . . . . C . . . . . G . . . . . -3'	46.2	2	100%
Rhinoceros sondaicus	Javan rhino	(RC 2)	5'- . . G . . . . C . . . . -3'	55.5	2	100%
Dicerorhinus sumatrensis	Sumatran rhino	(RC 3)	5'- . . . . C . . . . -3'	57.5	17	100%
Ceratotherium simum	White rhino	(RC 4)	5'- . . . . C . . T . . . . -3'	49.2	4	100%
Diceros bicornis	Black rhino	(RC 5)	5'- . . . . T . . . . C . . . . -3'	49.2	8	100%
Pangolin Probe		–	5'- G G A G G T T T* T A T G T T* A A T G A T A G T T* G T A A T -3'	–	–	–
Pangolin Reverse Complement Oligo		RC0	5'- A T T A C A A C T A T C A T T A A C A T A A A A C C T C C -3'	–	–	–
Manis pentadactyla	Chinese pangolin	RC1	5'- . . C . . . . C . . . . C . . . -3'	52.2	14	100%
Manis culionensis	Phillipine pangolin	RC2	5'- . . . . A . . . . . . C . . . . C . . . -3'	43.5	4	100%
Phataginus tricuspis	Tree pangolin	RC3	5'- . . . . T . . . . . . T . . . . -3'	48.2	27	89%
		RC6	5'- . . . . T . . . . . . T . . . . . . C . . . . . -3'	44.2		11%
Phataginus tetradactyla	Long-tailed pangolin	RC5	5'- . . . . A . . T . . . . . T . . . . -3'	44.2	15	73%
		RC6	5'- . . . . T . . . . . . T . . . . . . C . . . . . -3'	44.2		27%
Smutsia gigantea	Gaint pangolin	RC3	5'- . . . . T . . . . . . T . . . . -3'	48.2	6	100%
Smutsia temminckii	Ground pangolin	RC4	5'- . . C . . . . C . . . . -3'	53.2	8	100%

RC = Reverse complement oligo label. * In probe sequence denotes fluorophore position. "." Denotes same nucleotide as RC0 oligo. *Panthera* Forward Primer (Excess): 5'-TTGTTTACGGTCATGGCTACAGCCTT-3'; *Panthera* Reverse Primer (Limiting) 5'-GCTGACAGGAGGTTGGTGATTACG-3'; rhino Forward Primer (Excess) 5'-TTGACCTAACCATCTTCTCCCTACAC-3'; rhino Reverse Primer (Limiting) 5'-ATTGGGATATGGCTGGTGGTTT-3'; pangolin Forward Primer 1 (Excess) 5'-CCCTTCATTTGGCAGGTATCTCATCA-3'; pangolin Forward Primer 2 (Excess) 5'-CTCTCCACCTAGCAGGTATTTCCTCA-3'; pangolin Reverse Primer 1 (Limiting) 5'-CATACGAACAATGGGGTTTGGTATTG-3'; pangolin Reverse Primer 2 (Limiting) 5'-CATACAAATAGTGGAGTTTGATATTG-3'. *Manis javanica* and *Manis crassicuadata* not represented in the GenBank database

51 Results show a differentiation between target signal and mean background noise (Figure 1

52 a,c,e) which is supported by the results of a T-test showing a significant difference between

53 signal observed fluorescence in the negative control (NTC) and fluorescence for all target

54 species (*Panthera* P=<0.000; rhinoceros P=<0.000, pangolin P=<0.000). This suggests that the

55 probes do not form any secondary structures that may mask detection of target DNA. However,

56 The signal:noise ratio of each probe showed some marked differences in quality with both the

57 FAM labelled rhinoceros probe and the JOE labelled pangolin probe showing well defined

58 melt transitions and derivative peaks while the TAMRA labelled *Panthera* probe was less well

59 defined (Figure 1 a,c,e). Such observations have been previously reported for TAMRA labelled

60 HyBeacon probes and it is possible that an alternative probe label such at Cal-Fluor-610 would

61 allow better signal to noise while still being detected in a third channel (LGC, personal

62 communication).

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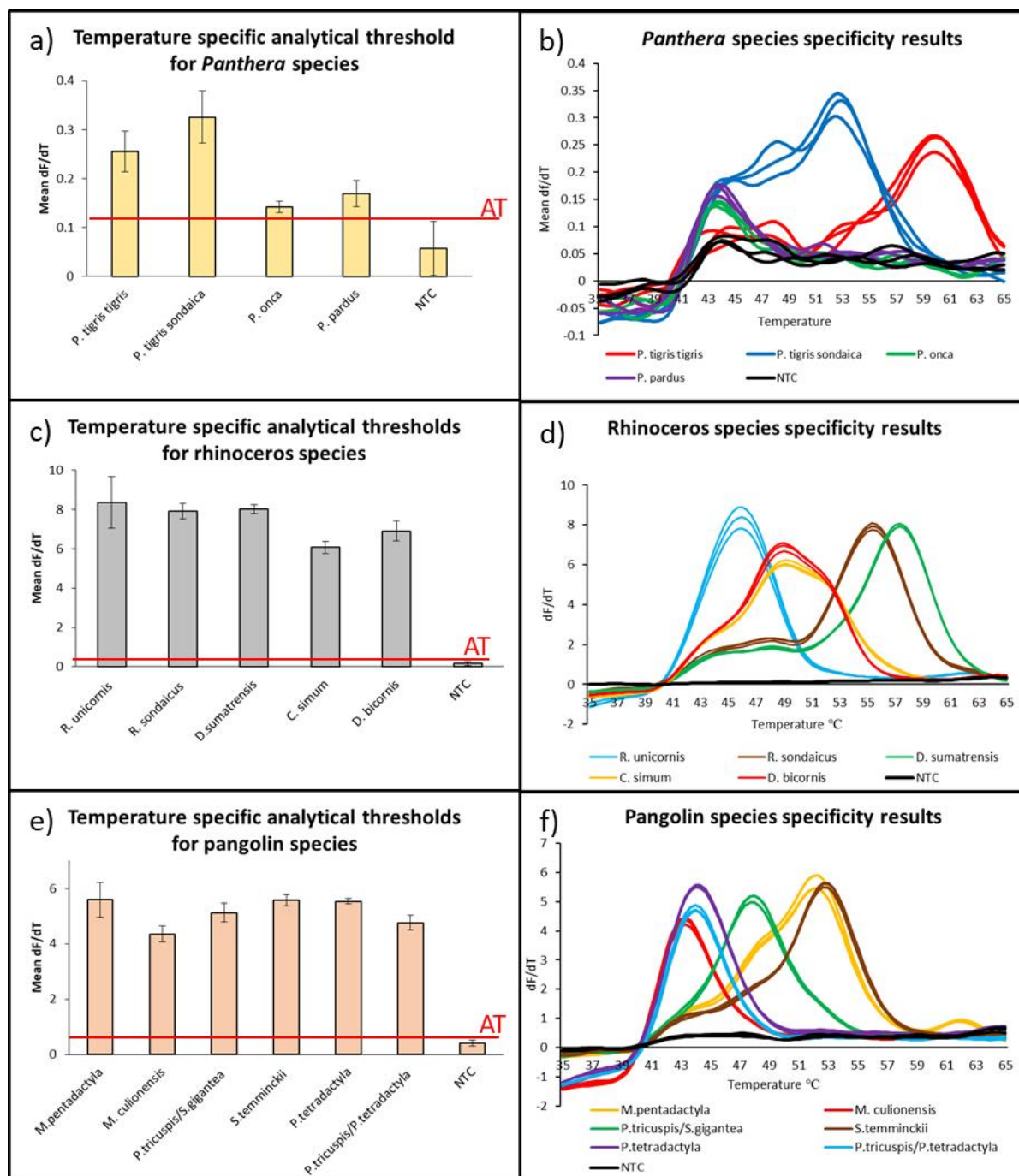
Figure 1. HyBeacon fluorescence data showing a) differentiation between four species signals and negative control (NTC) for TAMRA labelled *Panthera* probe; b) melt derivative peaks showing melt temperatures for TAMRA labelled *Panthera* probe; c) differentiation between five species signals and negative control (NTC) for JOE labelled rhinoceros probe; d) melt derivative peaks showing melt temperatures for JOE labelled rhinoceros probe; e) differentiation between six species signals and negative control (NTC) for FAM labelled pangolin probe; f) melt derivative peaks showing melt temperatures for FAM labelled pangolin probe. Error bars in a,c,e represent 3 Standard Deviation. AT = Analytical Threshold.

74 When assessing the utility of probes for wildlife forensic species detection it is important to

75 consider the protective legislation for the study species. Is species level identification required

76 or is genus level or family level identification sufficient? The latter example allows some

77 flexibility in probe design while the former example makes probe design more complicated.

78 The melt derivative peaks obtained from the *Panthera* probe (Figure 1b) show a clear

79 differentiation between the two tested tiger subspecies species with the tiger (*P. tigris tigris*)

80 showing a distinct peak at 59.8°C with the Sumatran tiger (*P. tigris sondaica*) showing a

81 distinct peak at 52.8°C. There were no temperature differences observed between the leopard

82 (*P. pardus*) and the jaguar (*P. onca*) with both species displaying a peak at 42.8°C. The inability

83 to differentiate between the African leopard and the South America jaguar is unexpected given

84 that there is an additional A/G transition observed in the leopard DNA sequence (Table 1).

85 Despite this, the probe shows utility for the investigation of IWT as all the target species are

86 CITES Appendix I listed and therefore illegal to trade [24]. The melt derivative peaks obtained

87 from the rhinoceros probe (Figure 1d) show a clear differentiation between the three Asian

88 species with Indian rhinoceros (*Rhinoceros unicornis*) showing a distinct peak at 46.2°C, the

89 Javan rhinoceros (*Rhinoceros sondaicus*) showing a distinct peak at 55.5°C and the Sumatran

90 rhinoceros (*Dicerorhinus sumatrensis*) showing a distinct peak at 57.5°C. There was no

91 difference in melt temperature between the two African species with the black rhinoceros

92 (*Diceros bicornis*) and the white rhinoceros (*Ceratotherium simum*) both showing a peak at

93 49.2°C. The lack of differentiation between the African species is due to them both sharing a

94 T/C transition albeit in a different place meaning there is no relative difference in melt

95 temperature. However, given all the species tested are CITES Appendix I listed and therefore

96 illegal to trade, the probe remains useful for the detection of the Rhinocerotidae family. The

97 rhinoceros data also highlights the potential to differentiate between the two geographic regions

98   (Asia and Africa) which, while not needed to level a criminal charge, may still provide useful

99   forensic intelligence to investigators attempting to understand IWT routes and the composition

100   of seizures [25]. Indeed, such work has been performed previously using microsatellite markers

101   to determine the geographic origin of large shipments of ivory [26,27]. While a tantalising

102   possibility, it is considered unlikely that there is sufficient variation in the mtDNA genome to

103   allow a finer 'population specific' origin to be determined using the approach described. The

104   melt derivative peaks obtained from the pangolin probe (Figure 1f) show a clear differentiation

105   between the two Asian species with Chinese pangolin (*Manis pentadactyla*) showing a distinct

106   peak at 52.5°C, and the Phillipine pangolin (*Manis culionensis*) showing a distinct peak at

107   43.5°C. Of the African species, only the ground pangolin (*Smutsia temminckii*) also showed a

108   species specific melt peak at 53.2°C, with the remaining species, tree pangolin (*Phataginus*

109   *tricuspis*), long-tailed pangolin (*Phataginus tetradactyla*) and the giant pangolin (*Smutsia*

110   *gigantean*) showing some shared melt peak temperatures at 44.2°C and 48.2°C. The inability

111   to differentiate between these remaining species does not invalidate the use of the probe for

112   supporting the IWT investigations as all the species are CITES Appendix I listed [28] so the

113   Manidae family detection remains useful.

114   The hypothetical Limit Of Detection (LOD) of the multiplex assay showed the LOD is 1000

115   copies of mtDNA for *Panthera* species (Figure 2a), 100 copies of mtDNA for rhinoceros

116   species (Figure 2b), and 10,000 copies of mtDNA for pangolin species (Figure 2c). It is

117   possible that the lack of sensitivity displayed by the pangolin species is due to the use of two

118   forward and two reverse primers, which were necessary given the diversity observed in the

119   aligned pangolin sequences. The use of multiple primer sets, designed in the same region,

120   increases the possibility that they may form hairpins effectively preventing them from

121   amplifying DNA. Using a conservative estimate of 1000 copies of mtDNA in each cell [29,30]

122 the level of sensitivity for the multiplex test is calculated at approximately 10 cells which

123 matches the sensitivity requirements of a rapid wildlife forensic field-test [31].

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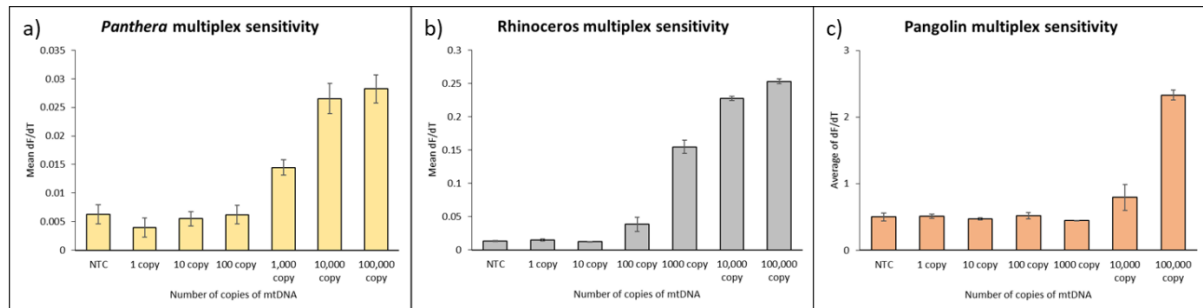
126 **Figure 2.** Sensitivity data showing the Limit of Detection (LOD) of the multiplex PCR assay

127 for a) *Panthera*, b) rhinoceros, and c) pangolin. Error bars represent 1 Standard Deviation.

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129 The data presented represents the first attempt to develop HyBeacon probes for the detection

130 of wildlife species subject to illegal trade and suggests that HyBeacon probes can offer

131 advantages over gel based identification techniques with regards to single step processing and

132 data quality. The probes described in the current study allowed the detection of individual

133 species in some instances but were limited to providing genus/family level detection in others.

134 However, given the protection status of the taxa described we believe that the probes described

135 could be used to screen and triage samples before sending for confirmatory forensic analysis,

136 an approach desired by wildlife forensic practitioners [31]. Further work could also look at the

137 development of a single test for each species, which would allow for the design of additional

138 species-specific probes based on other mtDNA regions which would allow species level

139 detection rather than the genus/family level detection described here. Such work should look

140 to design probes using an expanded mtDNA reference dataset, which limited the current study.

141 Indeed, the lack of reference sequence data meant that certain species were not considered at

142 all in the current study, including the Indian pangolin (*Manis crassicaudata*), the Sunda

143 pangolin (*Manis javanica*), the Bengal tiger (*Panthera tigris tigirs*), Malayan tiger (*Panthera*

144 *tigris jacksoni*), Siberian tiger (*Panthera tirgris altaica*), the Indochinese tiger (*Panthera tigris*

145 *corbetti*) and the South China tiger (*Panthera tigris amoyensis*). This is a problem that the

146 wildlife forensic community is attempting to solve through the development of a dedicated

147 database [32] which houses DNA sequence data from these forensically important species.

148 Once completed, it is likely that more researchers will be able to use the resource to help

149 develop molecular approaches to support wildlife forensic investigations leading to the

150 development of field-based assays for rapid sample screening at points of seizure [31]. Finally,

151 the development of wildlife forensic tests require extensive validation before application

152 [12,33,34] and further development and optimisation is necessary before the approach

153 described can be used in casework. Such work needs to increase the number of replicates used

154 across validation studies, transition from using synthetic DNA to extracted species DNA,

155 attempt detection of 'real world' samples, closely related 'non-target' species and mock

156 casework samples to ensure PCR amplification and HyBeacon detection is consistent and

157 reproducible. Future work will require collaboration with wildlife forensic laboratories to

158 ensure accurate and reproducible results are obtained before the probes are used in criminal

159 casework.

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