1   A TaqMan qPCR method for detecting *kdr* resistance in *Aphis gossypii* demonstrates

2   improved sensitivity compared to conventional PCR-RFLP

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19   **Abstract**

20   Cotton aphid, *Aphis gossypii* Glover, has emerged as a prominent pest in Australian cotton

21   production, monitoring pesticide resistance including pyrethroids in field populations is

22   crucial for its sustainable management. We examined the distribution of *kdr* resistance in 35

23   field collected *A. gossypii* populations and used a TaqMan qPCR assays with pooled samples.

24   The study demonstrated proof of concept that pooled insect qPCR methodology provided

25   effective detection with better sensitivity than individual PCR-RFLP genotyping techniques

26   for the *kdr* resistance allele. The practical outcome is that routine resistance monitoring can

27   examine more sites while increasing the likelihood of detecting incipient resistance at those

28   sites. More importantly, the method is adaptable to any genetically caused resistance and so

29   not limited to *A. gossypii* or even insect control. It cannot be overstressed that the ability to

30   detected resistance at very low frequencies is critical to all sustainable resistance

31   management. Early detection of resistance provides critical time for the modification of

32   chemical use prior to potential insecticide control failure.

33

36   **Key message**

37   • Cotton aphid has developed resistance to a range of insecticides in the Australian

38   cotton production system.

39   • There is a need for a quick and sensitive method of monitoring pyrethroid resistance

40   in field populations, particularly at low resistance allele frequencies.

41   •   We developed a TaqMan qPCR assay with pooled aphid samples with better

42   sensitivity than individual PCR-RFLP genotyping.

43   •   Our method can be adapted to any species and can examine more sites with increased

44   sensitivity of detecting resistance.

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Introduction

Worldwide, the cotton or melon aphid *Aphis gossypii* Glover is a major pest of many crops

causing damage by direct feeding and facilitating the transmission of plant viruses. In cotton,

significant crop value can also be lost from honeydew contamination of the open boll lint

(Herron et al. 2001). In Australian cotton, *A. gossypii* has emerged as a significant pest

requiring targeted chemical control (Herron et al. 2001). Counter intuitively this was due to

an overall reduction in insecticide use associated with transgenic *Bt*-cotton that controls the

Helicoverpa spp. and other lepidopteran insect species only (Herron and Wilson 2011).

Since *A. gossypii* has been targeted strategically for control it has developed resistance to a

range of insecticides including carbamates, endosulfan, organophosphates, pyrethroids

(Herron et al. 2001) and neonicotinoids (Herron and Wilson 2011) in both cotton and other

crops. In cotton, high-level resistance to the organophosphate (omethoate and dimethoate)

and carbamate (pirimicarb) insecticides in *A. gossypii* caused control failures due to an

insensitive acetylcholinesterase (*ACE1*) mediated resistance (Herron et al. 2001, McLoon and

Herron 2009). More recently, neonicotinoid resistance was detected in *A. gossypii* that again

caused control failures (Herron and Wilson 2011).

In Australian cotton, synthetic pyrethroids are not recommended for *A. gossypii* control

yet resistance to this insecticide group has been detected (Herron et al. 2001) and is likely

caused by concurrent or coincident selection where chemicals are applied to control other

pests when *A. gossypii* is also present (Herron and Wilson 2011). Increased metabolic

detoxification and decreased target site sensitivity (of the insect nervous system) are two of

the major mechanisms involved in the development of pyrethroid resistance in many pest

species. For instance, pyrethroid resistance due to metabolic detoxification has been reported

70 in fruit fly (Daborn et al. 2002), house fly (Zhu and Liu 2008), and green peach aphid (Silva

71 et al. 2012). Target site insensitivity of voltage gated sodium channel (VGSC) was first

72 identified in house fly (Williamson et al. 1993) due to a point mutation (Leu1014 to Phe)

73 within the domain IIS4–S6 region of the para-type VGSC gene. This is also termed

74 knockdown (*kdr*) resistance that causes DDT and pyrethroid resistance in insects and other

75 arthropods resulting from a reduced sensitivity in the sodium channels to the toxin. A second

76 mutation (Met918 to Thr) near to the S-4-S5 linker in domain II is known to cause much

77 higher resistance in house flies and so termed the *super-kdr* mutation (Williamson et al.

78 1996). The same *kdr* mutation cause pyrethroid resistance in malaria mosquito (Martinez-

79 Torres et al. 1998) and German cockroach (Miyazaki et al. 1996) and recently also in green

80 peach aphid (so potentially making it multiple resistant with two or more concurrent

81 mechanisms) (Martinez-Torres et al. 1999) and cotton aphid (Carletto et al. 2010). Although

82 pyrethroid resistance in *A. gossypii* in Australian was reported as early as 2001 (Herron et al.

83 2001) it was only a recent discovery that the mutation causing pyrethroid resistance in *A.*

84 *gossypii* was attributed to a target site mutation (L1041F) in the voltage gated sodium channel

85 (Herron et al. 2001, Marshall et al. 2012) and no *super-kdr* was observed.

86   Management of *A. gossypii* within all Australian cotton is achieved via a complex

87 integration of several techniques including specific agronomic practices to form an integrated

88 pest management (IPM) system (Herron and Wilson 2010). Resistance detection and ongoing

89 monitoring is essential to sustained IPM within agricultural production systems, with such

90 monitoring traditionally performed by bioassay (Busvine 1971). The method requires live

91 insects to be exposed to increasing concentrations of pesticide so that probit regressions can

92 be calculated and toxicity comparisons made, often at the LC_{50} level (a value that kills 50%

93 of treated insects) (Busvine 1971). This method, while accurate, can be tedious as the

94 bioassay requires the manipulation of live insects and strains are often laboratory maintained

95   (cultured) for a significant time before the bioassay can be conducted. Molecular methods to

96   detect resistance are becoming more normal (McLoon and Herron 2009) but those methods

97   may test a sample smaller than a conventional bioassay (Herron et al. 2001).

98   Mechanisms of pesticide resistance include increased production of insect metabolic

99   enzymes, modification of the pesticide target site by allele mutation or gene amplification

100   (Van Leeuwen et al. 2010, Bass and Field 2011). For target site resistance mutations, the

101   detection of resistance allele(s) in an insect population, can be achieved by molecular

102   methods including: allele specific PCR (Ranson et al. 2000, Liu et al. 2005); PCR restriction

103   fragment length polymorphism analysis (PCR-RFLP) (Daborn et al. 2004, Cassanelli et al.

104   2005, McLoon and Herron 2009, Marshall et al. 2012); high-resolution melt curve analysis

105   (Bass et al. 2007, Pasay et al. 2008); and several others (Kolaczinski et al. 2000, Lynd et al.

106   2005, Kulkarni et al. 2006). The resistance allele frequency is estimated by genotyping

107   multiple individuals (samples) from a field population. However, individual genotyping of

108   large numbers is not cost-effective and bias can arise in the estimate if only small numbers

109   are genotyped from any one field population. This is particularly a problem when the

110   resistance allele frequency in the population being tested is low.

111   As an alternative to individual genotyping, the resistance allele frequency can be estimated

112   by quantitative PCR (qPCR) TaqMan SNP assay using pooled samples (Yu et al. 2006, Chen

113   et al. 2014). Rapid identification of resistant alleles from pooled insect samples offers

114   significant throughput improvements for the detection of insecticide resistance and also

115   makes it easier to monitor large geographic areas.

116   We surveyed 35 *A. gossypii* field populations collected during the 2011-12 Australian

117   cotton season for *kdr* mediated pyrethroid resistance. Here we demonstrate a qPCR based

118   assay on pooled aphid samples as a sensitive and cost effective alternative to conventional

119   individual PCR-RFLP.

120   **Materials and Methods**

121   *Aphid Collection and maintenance*

122   *A. gossypii* strains were collected from commercial cotton and horticulture crops (Table 1) in

123   Queensland (Qld), New South Wales (NSW) and Western Australia (WA) and maintained as

124   live cultures on cotton (even if not originally collected from cotton) (Herron and Wilson

125   2011). Sampling was based on detailed aphid specific methods that required multiple

126   invested leaves to be collected from several locations (Wilson and Herron 2014). All strains

127   tested were collected during the 2011-2012 season and were maintained under insecticide

128   free conditions in a purpose built insectary isolated individually in insect proof cages (Wilson

129   and Herron 2014). Strain Mona Park was found susceptible and strain Territ resistant. Strain

130   Territ was then pressured on an *ad hoc* basis with 0.0003 mg active ingredient / L lambda-

131   cyhalothrin (pyrethroid) and several days before use as the resistant standard to eliminate the

132   possibility of reversion. Susceptible Mona Park was confirmed as such prior to use as a

133   reference susceptible via PCR-RFLP.

134   **< Table 1**

135   *Resistance allele frequency obtained with PCR-RFLP*

136   Samples were extracted within several weeks of collection months before reversion was

137   likely (Herron et al. 2001). Aphids used for PCR-RFLP were placed individually into

138   separate microcentrifuge tubes containing 80 μL of 5% Chelex-100 resin in Millipore water

139   solution. Aphids were thoroughly crushed using a sterile micropestle and microcentrifuge

140 tubes were heated to 56°C for 30 min, and then boiled at 100 °C for 5 min. The samples were

141 stored at -20°C until required. Aphid samples for qPCR were prepared as for PCR-RFLP as

142 above but with 200 aphids pooled into one microcentrifuge tube containing 500 µL of 5%

143 Chelex-100 resin solution.

144 PCR-RFLP was performed as previously described (Marshall et al. 2012). Briefly, primers

145 KDR-DP1 5'-TCTTGGCCCACACTTAATCTTT-3') and KDR-DP4 (5'-

146 CTCGCCGTTTGCATCTTATT-3) (Sigma-Aldrich, Castle Hill, Australia) were used to

147 amplify a 468 bp fragment, which contained the L1014F mutation. Restriction endonuclease

148 *Bst*EII (New England Biolabs, MA, USA) was added post-PCR and incubated at 60 °C for a

149 minimum of 6 hrs. Samples were visualised by gel electrophoresis using 2% agarose (Bio-

150 Rad) and stained with GelRed (Biotium Inc., CA, USA). Resistance allele frequency (RAF)

151 was calculated by genotyping 20 individual aphids for each population.

152 *Quantitative real-time PCR (qPCR)*

153 A TaqMan[®] assay was developed using forward primer 5'-

154 CCATTCTTCTTGGCTACTGTTGTCA-3' and reverse primer 5'-

155 CCCTAAGTAATACACATTTATGCATTGTCAGT-3' (Life Technologies Inc., CA, USA).

156 Dual-labelled probes 5'-FAM-CATACCACAAAGTTACC-3'-BQ1 and 5'-VIC-

157 CATACCACAAGGTTACC-3'-BQ2 (Life Technologies) were designed based on the

158 sequences of the previously described *A. gossypii* strains, those being Went F6 (resistant)

159 and SB (susceptible) (Marshall et al. 2012). PCR reactions contained 900 nmol forward

160 primer and reverse primer and 200 nmol susceptible and resistant probes, in 1 × TaqMan

161 Universal PCR Master Mix (Life Technologies) in a 25 µl reaction volume. Each sample was

162 set up in triplicate and one negative and positive control was included in each run. Real-time

163   PCR was performed in an ABI7500 Real-Time PCR System (Life Technologies) with 10 min

164   at 95ºC followed by 47 cycles of 15 s at 95ºC and then 1 min at 60ºC.

165

166   *Quantification of kdr resistance allele frequency*

167   As pyrethroid resistance in *A. gossypii* has previously been noted to be heterozygous

168   (Marshall et al. 2012), a standard curve for quantification of resistance allele frequency

169   (RAF) was generated by mixing susceptible and resistant aphids (total 20) with predefined

170   RAF's of 0.5, 0.475, 0.45, 0.4, 0.35, 0.3, 0.25, 0.2, 0.15, 0.1, 0.05, 0.025 and 0.

171

172   Resistance allele frequency was predicted on the transformed fluorescence ratio (k´) at the

173   inflexion point of a four parameter sigmoid curve (Chen et al. 2014). In brief, the transformed

174   fluorescence ratio k' is the transformation of the ratio of two fluorescence intensities, when

175   one fluorescence reaches its inflexion point. k' and four parameters of sigmoid curve, were

176   estimated by two-step sigmoid curve fitting, using raw fluorescence data. For each standard

177   point and unknown sample, k' was the average of the triplicated PCR reactions and the CV

178   was <5%. A prediction equation was built by using k' and standards of known RAF. As

179   described by Chen et al. (2014) there were four samples shared between runs for

180   normalization of multiple qPCR runs.

181

182   **Results**

183   *Pyrethroid resistance detected by PCR-RFLP*

184 Consistent with the previous study of Marshall et al. (2012) using PCR-RFLP, we found all

185 aphids with L10141F resistance allele to be heterozygous (Table 1). Of the six field

186 populations with resistant alleles, two were from NSW (Wisemantle and Wyadringah) and 4

187 from Qld (Alcheringa, Bore Paddock, Territ and Zagazig). In contrast, no resistance alleles

188 were detected in any WA collection (Table 1). Resistance allele frequency ranged from 0.05

189 in strain Wyadringah to 0.48 in strain Wisemantle. As *kdr* resistance in *A. gossypii* has

190 always been observed as heterozygous, a RAF of 0.48 equates to approximately 95% of

191 aphids tested being pyrethroid resistant.

192 *Pyrethroid resistance detected by qPCR with pooled aphids*

193 The prediction equation was based on a sigmoid function between transformed fluorescence

194 ratio (k´) and known RAF standard (Table 2). To evaluate the sensitivity of the qPCR assay,

195 we estimated the average standard deviation of the constructed RAFs three replicates to be

196 0.008 and subsequently set our sensitivity when testing field strains at a conservative 0.024.

197 Using this conservative level of significance we identified the L1014F allele in 5 additional

198 strains (Table 1). Additional strains identified as pyrethroid resistant included 3 from NSW

199 (Carnarvon F3, Carrington and Springfield) and 2 from Qld (Balondale F3 and Boonal

200 Dryland). Furthermore, strain Burgorah Clo from Qld was identified as pyrethroid resistant

201 by qPCR while the PCR-RFLP genotyping failed. Unfortunately, PCR-RFLP analysis could

202 not be repeated on this strain due to culture failure.

203 **< Table 2**

204 **Discussion**

205 Pyrethroid use in Australian cotton was once a mainstay choice for insect control until

206 insecticide resistance was linked to control failure during 1983 in the major Australian cotton

207   pest species *Helicoverpa armigera* (Hübner) (Gunning et al. 1984). Resistance management

208   based on chemical alternation and use restrictions was implemented slowing pyrethroid

209   resistance but frequencies gradually increased until industry was on the verge of crisis

210   (Forrester et al. 1993). It was not until the introduction of transgenic *Bt*-cotton in 1996 (that

211   controlled *H. armigera*) were Australian cotton growers truly able to embrace IPM as a

212   control tactic that eventually resulted in a substantial 85% reduction in pesticide use

213   (Constable et al. 2011). Ironically, that decline in insecticide use in *Bt*-cotton allowed

214   secondary pests such as whitefly, mirids, and aphids to survive, that when targeted for

215   control, again caused resistance (Herron et al. 2001, 2011).

216   Pesticide resistance monitoring is an integral part of the successful maintenance of IPM of

217   *A. gossypii* in Australian cotton (Mass et al. 2014). Despite pyrethroids not being specifically

218   targeted against *A. gossypii* in Australian cotton, pyrethroid resistance is well established in

219   *A. gossypii*. Pyrethroid resistance in Australian *A. gossypii* was detected via bioassay and it

220   was not until very recently that molecular based methodology was developed to detect the

221   *kdr*-associated L1014F polymorphism (Marshall et al. 2012). The reasons for the

222   polymorphism being common in Australian *A. gossypii* are complex and probably include

223   non-cotton plant species such as melons hosting *A. gossypii* that do receive pyrethroid sprays

224   for *A. gossypii* control. In fact, a recent genetic structure study of *A. gossypii* in Australia

225   showed weak host plant specialisation and many plant species could serve as refuge plants for

226   *A. gossypii*.(Chen et al. 2013). However, it is also likely that the *kdr* resistance detected in *A.*

227   *gossypii* is maintained by the use of pyrethroids targeting other insect pest species in cotton

228   with *A. gossypii* experiencing co-incidental or concurrent selection (Herron and Wilson

229   2011).

230   By using qPCR rather than PCR-RFLP we were able to identify 4 additional *A. gossypii*

231   strains exhibiting low-level *kdr* resistance which were not previously detected. We consider

232   the small sample-size of the individual genotyping PCR-RFLP method is the most likely

233   reason for the difference in sensitivity in population level between methods, especially when

234   the resistance allele frequencies fall below 5%. On average, under the RFLP-PCR scenario,

235   only 2 aphids will be carrying the resistance mutation in a 20 aphid sample so the likelihood

236   of a false negative is high. Therefore when the resistance allele frequency is low, the qPCR

237   with a pool of 200 aphids provides a more robust and sensitive prediction. In addition, the

238   pooled qPCR method reduced the number of PCRs required to estimate the resistance

239   frequency for each field population. Hence it is possible to monitor more sites/areas as the

240   principal cost of these techniques is PCR-based reagents. Cost-effective and accurate

241   methods could potentially allow more frequent resistance monitoring and increase the

242   likelihood of detecting incipient resistance.   In addition to the proof of concept that qPCR

243   improves the chance of resistance detection in *A. gossypii* (or any other species), the ability to

244   significantly and cost-effectively increase sample size tested has profound ramifications for

245   resistance management generally. For example, Australian cotton (and many other

246   agricultural systems) relies heavily on the glyphosate herbicide for weed control and the

247   threat of resistance to it has caused an integrated pest management system to be implemented

248   (Mass et al. 2014). The qPCR methodology described here is adaptable to glysophate and so

249   can be used to monitor glysophate resistance in weeds associated with cotton. It cannot be

250   overstressed that the ability to detected resistance at very low frequencies is fundamental to

251   sustainable management because it can give early warning prior to failures and provide

252   critical time to modify chemical use within the management system. For a sustainable

253   integrated pest management system, we recommend the use of qPCR routinely to monitor *kdr*

254   resistance. However, it is still desirable to occasionally perform complementary pyrethroid

255 bioassay as the current qPCR assays only detect the known L1014F *kdr* mutation and

256 pyrethroid resistance can arise from increased detoxification enzyme activity or other

257 mutations in *VSSC* gene. For that reason it would also be desirable to sequence the VSSC

258 gene if pyrethroid resistance is detected via bioassay if qPCR did not detect the *kdr* resistance

259 allele. This procedure will identify if there is a new mutation in the VSSC gene or mutations

260 in the primer region which can be beyond the sensitivity of the current qPCR method.

261 Further, the DNA extract from 200 aphid can be used for detect other resistance alleles such

262 *super-kdr*.

263   In conclusion, the *kdr* resistance allele appears widespread in both Australian horticultural

264 and cotton growing regions with resistance frequencies in individual strains ranging from low

265 to high. Resistance management implications are significant because cotton in the vicinity of

266 sprayed horticulture may be negatively affected by those sprays while the inadvertent co-

267 incident selection of *A. gossypii* within cotton may again adversely affect future control

268 options. Quantitative PCR from pooled aphid samples can be used to monitor pyrethroid

269 resistance in field populations in support of IPM with improved precision compared to PCR-

270 RFLP. The methodology is not limited to aphids or even insects but adaptable to any species

271 that develops resistance.

272 Author contributions

273 YC GH and SM conceived and designed the experiments. MS and DB performed the

274 experiments. IB and YC analyzed the data. GH contributed reagents/materials/analysis tools

275 via DAN1203. MS, DB, YC and GH wrote the paper. All authors have read and approve the

276 manuscript.

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Table 1
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Table 1. Resistance allele frequency (RAF) in the 2011-2012 Australian cotton season

Strain	Location	Crop	RAF (RFLP)[1]	k'	RAF (qPCR)
Carnarvon F3	northern inland, NSW	Cotton	0.00	0.164	**0.032***
Carnarvon Vol.	northern inland, NSW	Cotton	0.00	0.118	0.015
Carrington	northern inland, NSW	Cotton	0.00	0.179	**0.035***
Springfield	northern inland, NSW	Cotton	0.00	0.162	**0.031***
Wisemantle	northern inland, NSW	Cotton	**0.48**	0.565	**0.485**
Wyadringah	northern inland, NSW	Cotton	**0.05**	0.148	**0.025**
Alcheringa	Darling Downs, QLD	Cotton	**0.08**	0.134	0.020
Araluken	Darling Downs, QLD	Cotton	0.00	0.103	0.011
Arrawatta	Darling Downs, QLD	Cotton	0.00	0.136	0.021
Boonal Dryland	Darling Downs, QLD	Cotton	0.00	0.179	**0.038***
Boonal Irrigation	Darling Downs, QLD	Cotton	0.00	0.110	0.013
Budleah	Darling Downs, QLD	Cotton	0.00	0.108	0.013
Eumorella	Darling Downs, QLD	Cotton	0.00	0.132	0.020
Fairview	Darling Downs, QLD	Cotton	0.00	0.119	0.016
Overflow	Darling Downs, QLD	Cotton	0.00	0.120	0.016
Territ	Darling Downs, QLD	Cotton	**0.38**	0.540	**0.463**
Waltons	Darling Downs, QLD	Cotton	0.00	0.106	0.012
Anderson	Fitzroy, QLD	Cotton	0.00	0.122	0.017
Griar 148	Fitzroy, QLD	Cotton	0.00	0.127	0.018
Mona Park	north QLD	Cotton	0.00	0.111	0.013
Balondale F3	south west QLD	Cotton	0.00	0.151	**0.025***
Balondale Vol.	south west QLD	Cotton	0.00	0.084	0.007
Bore Paddock	south west QLD	Cotton	**0.08**	0.263	**0.098**
Brookfield Clear	south west QLD	Cotton	0.00	0.133	0.020
Brookfield Trees	south west QLD	Cotton	0.00	0.126	0.018
Burgorah Clo.	south west QLD	Cotton	-	0.241	**0.078***
Burgorah Dry	south west QLD	Cotton	0.00	0.128	0.018
Clyde	south west QLD	Cotton	0.00	0.124	0.017
Doondi 1	south west QLD	Cotton	0.00	0.112	0.014
Doondi 2	south west QLD	Cotton	0.00	0.119	0.016
Zagazig	south west QLD	Cotton	**0.06**	0.267	**0.102**
Bothkamp	Kimberley, WA	Rockmelon	-	0.115	0.015
Bothkamp B	Kimberley, WA	Pumpkin	0.00	0.123	0.017
Chilman	Kimberley, WA	Zucchini	0.00	0.132	0.020
Pacific Seeds	Kimberley, WA	Cotton	0.00	0.116	0.015
Tropical Sands	Kimberley, WA	Watermelon	0.00	0.132	0.020
Wanhoe Farms	Kimberley, WA	Pumpkin	0.00	0.121	0.016

1: all *kdr* positive samples genoyped by PCR RFLP were heterozygous wt/kdr

- Culture failed before PCR-RFLP analysis could take place.

Bold font in the RAF column indicates that the field population has the *kdr* resistance allele present.

*resistance allele frequency detected by qPCR with 200 pooled aphids, but no resistance allele was observed by genotyping 20 aphids by individual PCR-RFLP. Delineation of resistance was considered positive if the predicted RAF was within 3 times the standard deviation of the triplicate produced by the susceptible reference strain only (1x sd = 0.024 so 3x 0.008). Using this estimate further investigation is needed if an absolute zero allele frequency is required.

Table 2
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Table 2. Transformed fluorescence ratio (k´) and known RAF standard.

Standard/Sample	transformed fluorescence ratio (k')	CV (%)
RAF 0.50	0.577*	2.16
RAF 0.475	0.557	1.26
RAF 0.45	0.533	1.66
RAF 0.40	0.481	1.68
RAF 0.35	0.443	0.66
RAF 0.30	0.410	3.20
RAF 0.25	0.386	1.15
RAF 0.20	0.348	3.26
RAF 0.15	0.315	1.40
RAF 0.05	0.169	1.80
RAF 0.025	0.147	0.74
RAF 0.00	0.105	4.69

Prediction equation

$$y = 0.5856 / (1 + EXP(-(k' - 0.401)/0.0945)) - 0.0126$$

$R^2 = 0.99$

*: the average transformed fluorescence ratio (k´) of triplicates

CV: coefficient of variation of transformed fluorescence ratio (k´) of triplicates