Species:	*Pyrenophora tritici-repentis*
Product Class(es):	Qo-Inhibitors
Method type described:	Molecular genetic detection of mutations conferring QoI resistance in *Pyrenophora tritici-repentis* in wheat leaves.
Date of protocol:	2015-05
Version	1
comments	Proven for the mutations F129L, G137R and G143A

Method:

Pyrosequencing is a unique detection technology based on the principle of sequencing-by-synthesis. Mutations conferring fungicide resistance can be detected qualitatively and quantitatively with this method in fungal pathogens. The detection limit of mutations in DNA samples from fungal populations is ~5-10 %.

1. Sampling and DNA extraction: From each sample 20 lesions from dried wheat leaves with *Pyrenophora tritici-repentis* symptoms are cut out and used for DNA extraction. DNA is extracted using Nucleo Spin Plant Kit (Macherey-Nagel) following manufactures' instructions.

2. PCR and Pyrosequencing: In a first step cytochrome *b* gene fragments containing the target sequences are amplified in PCR reactions with a final volume of 25 µl using 12.5 µl 2x Maxima Mastermix (Fermentas), 1.25 µl of each primer for amplification of codon 129 (KES 432: 5´ TCCTAACTTAAAAGGTTACACAAGGCTT 3´ and KES 433: 5´ Biotin-AACCATTTTGGGCTATGTTGGTA 3´ final concentration 500 nM) and codon 137-143 (KES 610: 5´Biotin- TTAGCCTGATATTTGGTCACAAGA 3´ and KES 611: 5´ TCGCCCTTTTTAACTGTAGCA 3´;), respectively, 7.5 µl bidest water and 2.5 µl DNA under the following conditions: An initial heating step for 4 min at 95°C is followed by 40 cycles with 15 sec at 95°C, 30 sec at 55°C and 15 sec at 72°C and with a final step of 5 min at 72°C. The subsequent pyrosequencing reactions of codons 129, 137 and 143 in these PCR products are performed using the specific sequencing primer KES 434: 5´

CGGAACTTAGACAGCC 3´for sequencing of codon 129 and KES 611: 5´ TCGCCCTTTTTAACTGTAGCA 3´ for sequencing of codons 137 -143 following the manufactures' instructions.

3. Qualitative and quantitative analysis: Pure isolates (genetic clones) can be analysed qualitatively (wild type or mutation) by pyrosequencing and reading the pyrograms. Quantitative analyses of mutations in populations are done with PSQ96MA Software. The possible nucleotide exchanges for F129L, G137R and G143A must be known for quantification of mutations (see Table 1), since this information is necessary for running the PSQ96MA software.

Table 1: List of wild type and mutated codons. Codons for mutations are those which are possible by a single nucleotide exchange of the wild type codon.

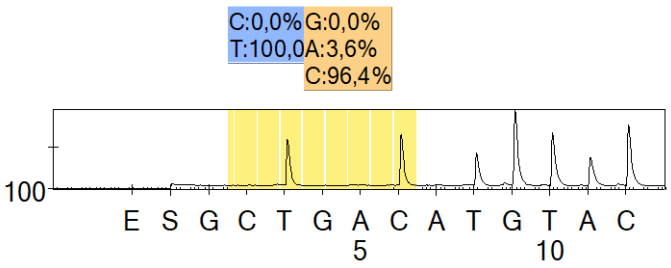
Codon	Wild type	Mutation
129	TTC	CTC TTA TTG
137	GGG	AGG
143	GGT	GCT

Since the F129L has been detected with 3 different codons (TTA, TTG and CTC) the allele frequencies of these codons have to be measured and counted up to a final F129L value (single values should be >10%).

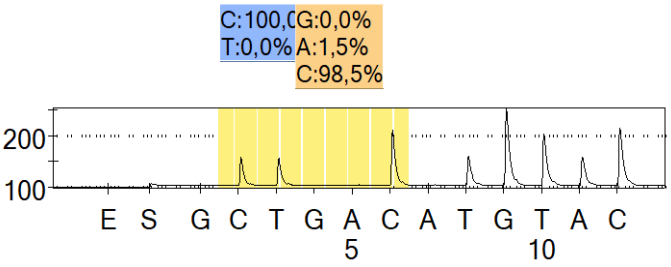
4. Examples

Example for F129L

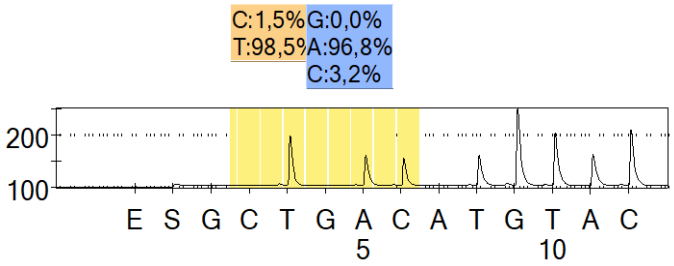
100% Wildtyp



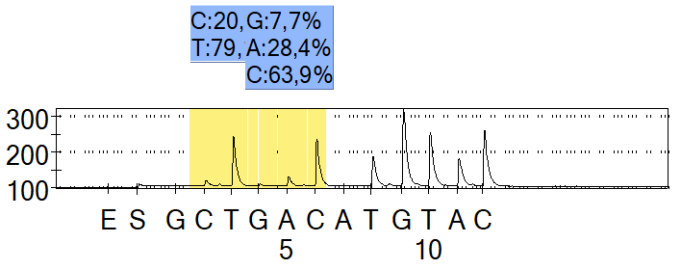
100% F129L (Codon: CTC):



96.8% F129L (Codon: TTA)

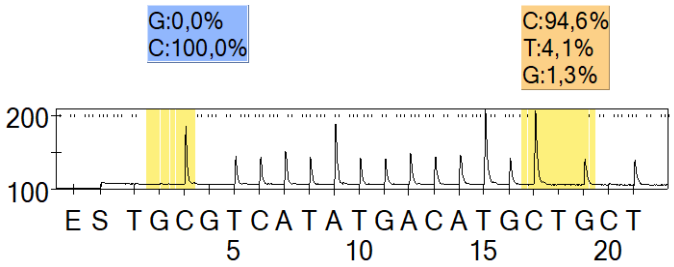


48% F129L (Codon CTC 20% + Codon TTA 28%)

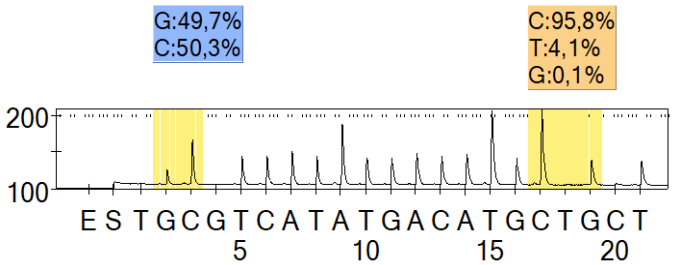


Example for G143A and G1437R

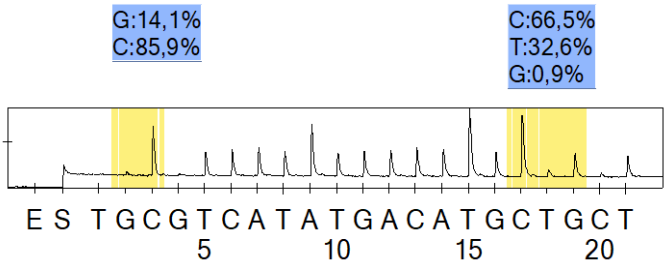
100% G143 (Wildtyp) und 94.6% G137 (Wildtyp):



49.7% G143A und 95.8% G137 (Wilytyp):



14.1% G143A und 32.6% G137R:



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