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

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 barley leaves with *Rhynchosporium secalis* 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 a cytochrome *b* gene fragment containing the target sequences is amplified in a PCR reaction with a final volume of 25 µl using 12.5 µl 2x Maxima Mastermix (Fermentas), 1.25 µl of each primer (KES 494: 5´ CGGATCATATAGAGCACCTAGAA 3 and KES 495: 5´ Biotin- TTATTAACAGAAAAACCCCCTCAG 3´; final concentration 500 nM), 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 this PCR product are performed using the specific sequencing primer KES 496: 5´ ATATTAATGATCGTTACAGC 3´ for sequencing of codons 129 and 137 and the primer KES 497: 5´ TTATGGACAGATGTCTTTAT 3´ for sequencing of codon 143 following the manufactures' instructions.

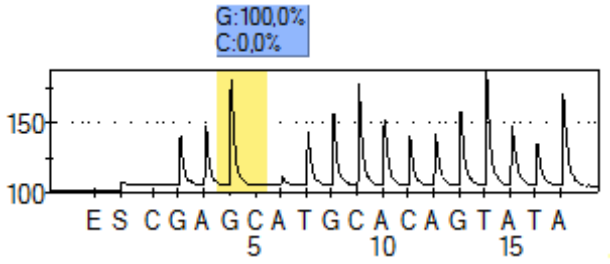
3. Qualitative and quantitative analysis: Pure isolates (genetic clones) can be analysed qualitatively (wild type or mutation) by pyrosequencing and reading the pyrograms. Quantitative analyses of mutations in populations are done with PSQ96MA Software. The possible nucleotide exchanges for F129L, G137R 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.

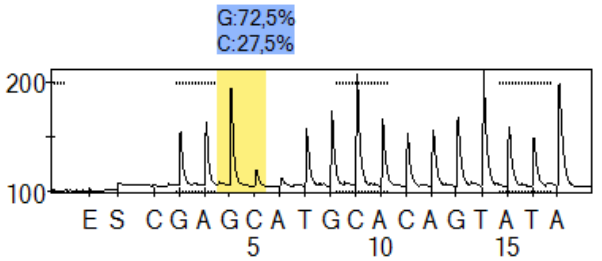
Codon	Wild type	Mutation
129	TTC	CTC TTA TTG
137	GGA	CGA AGA
143	GGT	GCT

4. Examples

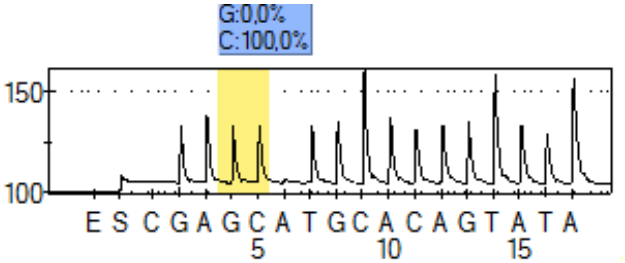
0% G143A



27.5% G143A



100% G143A



authors	Simone Miessner, Andreas Koch, Dr. Gerd Stammler, BASF SE, 67117 Limburgerhof, Germany gerd.stammler@basf.com