

Multiplex real-time single nucleotide polymorphism detection and quantification by quencher extension

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*Multiplex quencher extension (multiplex-QEXT) is a novel closed tube single-step method for detection and quantification of several single nucleotide polymorphisms (SNPs) simultaneously. The principle of multiplex-QEXT is that 5' reporter-labeled probes are 3' single-base-extended with TAMRATM dideoxy nucleotides if the respective SNP alleles are present. TAMRA can serve as either an energy acceptor (quencher-based detection) or donor [fluorescence resonance energy transfer (FRET)-based detection] for a wide range of different reporter fluorochromes. The extension can therefore be recorded by the respective reporter fluorescence change. We evaluated multiplex-QEXT, analyzing four different SNP loci in the *Listeria monocytogenes* inlA gene. Probes labeled with the reporters 6-FAMTM, TETTM, VICTM, and Alexa Fluor[®] 594 were used. Responses for the fluorochromes 6-FAM, TET, and VIC were detected by quenching (decreased fluorescence), while the response for Alexa Fluor 594 was detected by FRET (increased fluorescence). We evaluated the SNP-allele pattern in 252 different *L. monocytogenes* strains. Multiplex-QEXT gave a good resolution, detecting seven major and five minor groups of *L. monocytogenes*. Comparison with serotyping showed that multiplex-QEXT gave better resolution. We also evaluated the quantitative aspects of multiplex-QEXT. Quantitative information was obtained for all the fluorochrome/probe combinations in the sample pools. The detection limits for 6-FAM, TET, and Alexa Fluor 594 were the presence of the 10% target SNP alleles ($P < 0.05$), while the detection limit for VIC was the presence of the 5% target SNP alleles ($P < 0.05$). Currently, overlap in the fluorescence emission spectra is the limiting factor for the multiplexing potential of QEXT. With the emergence of new fluorochromes with narrow emission spectra, we foresee great potential for increasing the multiplex level in the future.*

INTRODUCTION

We have developed multiplex quencher extension (multiplex-QEXT), which is a novel method for detection and quantification of several single nucleotide polymorphisms (SNPs) simultaneously. The multiplex-QEXT detection is done in real-time by direct measurements of fluorescence changes in a closed tube system. The principle of multiplex-QEXT is that 5' reporter-labeled probes are 3' single-base-extended with TAMRATM-labeled dideoxy cytosine if the respective SNP alleles are present. TAMRA can serve as either a fluorescence energy acceptor or donor, depending on the 5' fluorescent reporter. The extension results in increased reporter fluores-

cence by fluorescence resonance energy transfer (FRET) if TAMRA serves as an energy donor, while if TAMRA is an acceptor, then the reporter fluorescence will be quenched. The multiplex-QEXT principle is shown schematically in Figure 1.

There is a continuous development and improvement of SNP detection technologies (1–4). Three general strategies have been used to increase efficiency and throughput. Increased throughput has been obtained by pooling the samples and analyzing SNP frequencies in the sample pools (1,5,6). Another strategy has been to develop real-time approaches that require very little sample handling (1,5,7–10). Finally, multiplex detection of several SNP alleles simultaneously from the

same sample is commonly done to increase throughput (11–13). To our knowledge, no assay has yet exploited the combined benefit of the three strategies mentioned above (14). The aim of our work was to develop an assay that enables the integration of all the above-mentioned approaches.

Typing of *Listeria monocytogenes* was used as a model to demonstrate the application of multiplex-QEXT. This pathogenic bacterium represents a major problem for modern food production (15). *L. monocytogenes* is often persistent in food and food production environments, but only a few types are pathogenic to humans (16). There is, however, a very serious outcome of listeriosis, with a mortality rate above 30%. Determining the reservoirs and understanding food contamination are very important in combating this bacterium (15).

Multi-locus sequence typing (MLST) is emerging as an alternative technique for bacteria typing (17). MLST is based on the DNA sequencing of several different loci in each bacterium analyzed. The identified SNP alleles are then used for the bacterial typing. DNA sequencing, however, severely limits the throughput of MLST. Furthermore, most of the DNA sequence information is redundant. Recently, typing techniques that only target the informative SNP alleles have been developed (1,10). What is lacking, however, are techniques that enable high-throughput analyses of several SNP loci simultaneously.

Here, we demonstrate the high-throughput application of multiplex-QEXT by the detection of four different SNP loci in 252 *L. monocytogenes* strains. We present data that show the discriminatory power, reproducibility, and quantitative properties of multiplex-QEXT.

MATERIALS AND METHODS

Bacterial Strains and QEXT Template

We analyzed 252 *L. monocytogenes* strains (listed in Supplementary Table S1, available online at www.BioTechniques.com). The strains

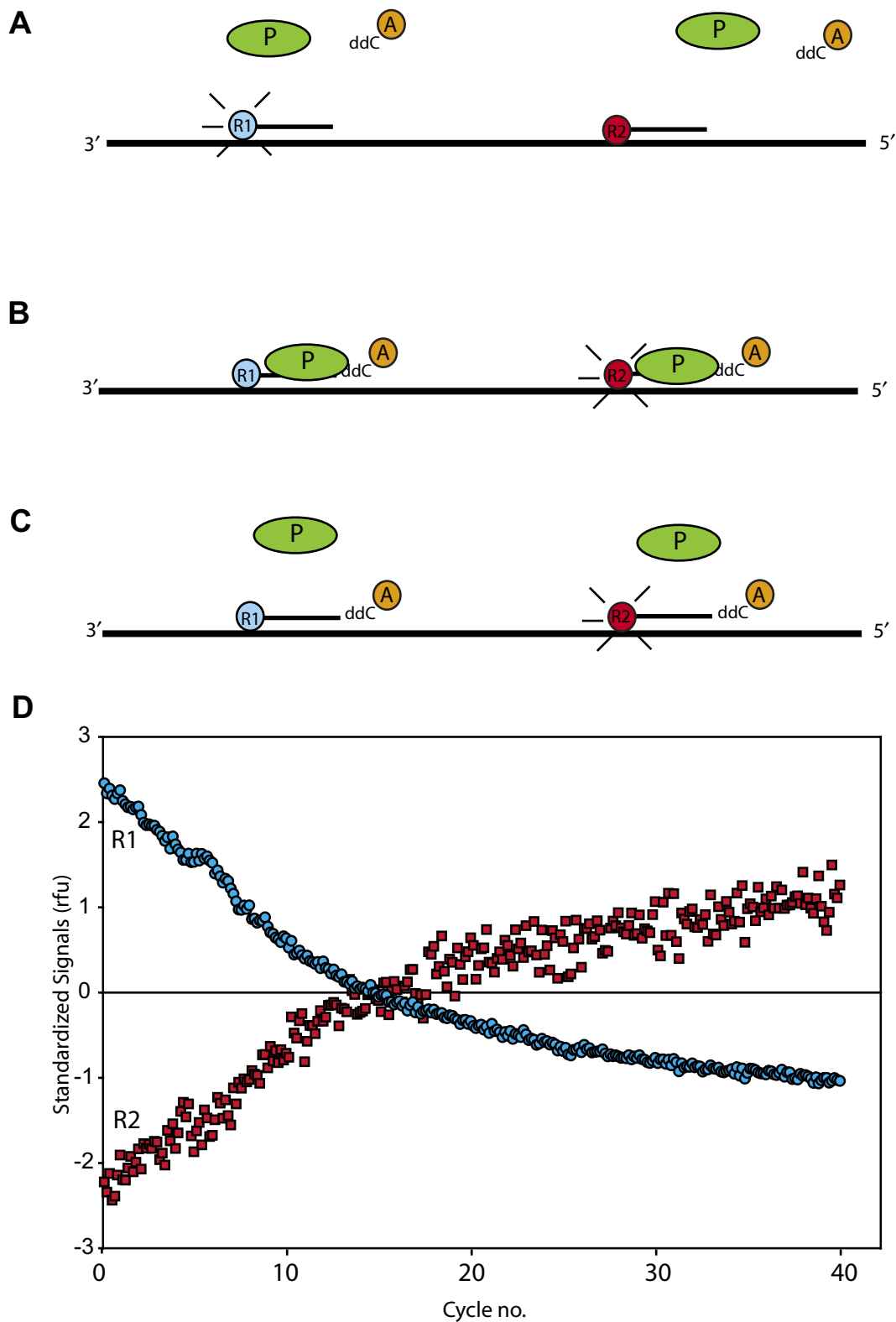


Figure 1. Representation of the multiplex quencher extension (multiplex-QEXT) principle. DNA is shown as black bars, the DNA polymerase as green ovals with the P inside, the dideoxycytosine labeled with an energy acceptor is shown as ddC with an orange circle with an A inside, and the reporters are shown as blue and red circles with R1 and R2 inside, respectively. (A) R1 emits fluorescence, while R2 does not emit fluorescence before incorporation of ddCTP. (B) The DNA polymerase incorporates ddCTP if the target single nucleotide polymorphism (SNP) alleles are present. (C) The emitted fluorescence from R1 is quenched, while the fluorescence for R2 increases [due to fluorescence resonance energy transfer (FRET)] after the sequence-specific incorporation of ddCTP. (D) Results from a sample containing both the R1 and R2 target mutations are shown for R1 (blue circles) and R2 (red squares). The R1 fluorochrome is TET, and the R2 fluorochrome is Alexa Fluor 594 in this example. rfu, relative fluorescence units.

were grown overnight in trypton soya broth (TSB) at 30°C, and DNA was purified as previously described (10). All positions of primers, probes, and products are given relative to an *inlA* reference sequence (GeneBank® accession no. AF497167).

We used the same approach for PCR amplification and pretreatment of the QEXT template as reported for the simplex QEXT assay (1). Briefly, the protocol involves amplifying the *inlA* gene (position 738 to 1483) with the primers InA-F (5'-GGAGCTAACCAATAAGTAACATCAGT-3') and InA-R (5'-TATCCGTACTGAAATCCATTTAGTT-3') and treating the amplified products with shrimp alkaline phosphatase and exonuclease I to inactivate the nucleotides and to degrade residual PCR primers. The modifications we made were to increase the primer concentration from 0.2 to 1 μM and to increase the exonuclease I/shrimp alkaline phosphatase treatment time from 30 min to 1 h.

Multiplex QEXT Reaction

We used the following detection probes in the multiplex-QEXT assay of detecting *inlA* SNPs: SNP position 1331 by InlA1 (5'-CGCAGCCACTTAAAGCAATTTTAAATG-3'), SNP position 1023 by InlA2 (5'-TCGGCTGGGCATAACCAAATTAGCGA-3'), SNP position 867 by InlA3 (5'-GGCTTATATCACTTATATTATTAAGTACA-3'), and SNP position 1299 by InlA4 (5'-GGAAAAGGAACGACAACATTTAGTGGAAAC-3'). The probes were 5'-labeled with the following reporter fluorochromes: 6-FAM™, Alexa Fluor® 594, TET™, HEX™, VIC™, Texas Red®, and Cy™5. All primers were high-performance liquid chromatography (HPLC)-purified. The probes have been designed following standard primer design (Primer Express 2.0; Applied Biosystems, Foster City, CA, USA).

The 10-μL multiplex-QEXT reaction contained 0.6 μL Concentrated ThermoSequenase™ Reaction Buffer (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), 100 μM TAMRA-labeled dideoxy cytosine nucleotides (TAMRA-ddCTP; Perkin Elmer, Boston, MA, USA), 0.1 μL Thermo Sequenase (32 U/μL;

Amersham Biosciences), and 2 μL PCR product treated with exonuclease I and shrimp alkaline phosphatase.

The thermal cycling was done using an ABI PRISM® 7900 HT sequence detector (Applied Biosystems). We used a thermal profile with an initial denaturation at 95°C for 2 min, and then cycling for 40 cycles with denaturation at 95°C for 30 s and an extension at 55°C for 1 min. The data collection was done at the extension stages during the thermal cycling.

Decomposition of the Multiplex-QEXT Signals

We used the multicomponent analysis in the Sequence Detection System (SDS) 2.2 software (Applied Biosystems) for obtaining information about the pure spectra for the different fluorescence components in the multiplex-QEXT reaction. Reference spectra for the dyes Texas Red, Alexa Fluor 594, and Cy5 were created following the instructions for the SDS 2.2 software, while the reference spectra for TAMRA, 6-FAM, TET, HEX, and VIC were included by the manufacturer. The data from the decomposed spectra were exported in a tab-delimited text format for further analyses.

Determination of Probe-Specific TAMRA-ddCTP Incorporation

The TAMRA-ddCTP incorporation rate per cycle was determined by regression analyses (see the supplementary material for details). The regression was done based on the multicomponent output from the SDS 2.2 software. We developed a Microsoft® Excel® Visual Basic macro for this analysis (see the supplementary material for details). The Delta outcome option in the macro and 25 cycles were used in the regression analyses.

Categorical Analyses of SNP-Allele Patterns

Categorical SNP allele patterns were determined using strains with known SNP patterns for calibrating the classification model. We allowed an approximately 0.5-fold fluctuation in

the amount of template (due to differences in PCR amplification) in the classification model. The amount of PCR product template was determined by agarose gel electrophoresis using ethidium bromide staining in combination with laser scanning for detection (Typhoon™ 8600 variable mode imager; Amersham Biosciences). The subsequent PCR product quantification was done using the ImageMaster™ Total Lab software (Amersham Biosciences) by comparison with a known DNA standard (molecular weight standard VI; Hoffmann-La Roche, Basel, Switzerland).

We used a $P = 0.05$ confidence threshold of the nontarget SNP allele distributions for scoring the absence of SNP alleles in strains with unknown SNP allele patterns. The remaining SNP alleles were scored as present. We chose the nontarget distribution for SNP allele scoring because this distribution is not dependent on the allowed fluctuations in the amount of target.

Quantitative SNP Analyses

Standard curves for the quantitative SNP analyses were made by mixing PCR products containing the SNP alleles with products without the SNP alleles in known ratios. Linear regression (Microsoft Excel) under the least square optimization criterion was subsequently used to determine the correlation between incorporation rates and amount of SNP target alleles.

The detection limit of the assay was determined by two-tailed Student's *t*-tests (MINITAB® Release 14; Minitab, State College, PA, USA). The detection limit was set to the lowest concentration of SNP alleles for which a significant difference ($P < 0.05$) in incorporation rates could be determined compared with control samples containing nontarget SNP alleles.

RESULTS AND DISCUSSION

Fluorochrome Compatibility in Multiplex-QEXT

The reporter fluorochromes 6-FAM, TET, HEX, VIC, Alexa Fluor 594,

Table 1. Evaluation of Reporter Signal-to-Noise Ratios

Reporter	Energy Transfer ^a	Excitation (nm)	Emission (nm)	Significance ^b (P < 0.01)
6-FAM	acceptor	490	520	yes
HEX	acceptor	535	553	yes
TET	acceptor	521	538	yes
Alexa Fluor 594	donor	594	617	yes
Texas Red	donor	593	612	yes
VIC	acceptor	538	554	yes
Cy5	donor	650	667	no

^aTAMRA serves as either an energy acceptor or donor, depending on the reporter fluorochrome.
^bThe significance in the signal was determined by two-tailed Student's *t*-test for the signals before labeling and after 25 cycles of labeling for 200 nM probe and approximately 75 ± 25 nM target. The FAM signal was evaluated for all the probes applied in this work, while TET and HEX were evaluated for InIA1, Alexa Fluor 594 for InIA3, Texas Red and VIC for InIA2, and Cy5 for InIA 4.

Texas Red, and Cy5 were evaluated with respect to the suitability for multiplex-QEXT. We first determined the QEXT signal-to-noise ratio for the different reporter fluorochromes. This was done by a two-tailed Student's *t*-test, analyzing the difference in signals before and after the QEXT labeling. All fluorochromes except Cy5 gave significant QEXT responses (Table 1). The failure to detect a QEXT response for Cy5 is probably because this FRET detection requires close proximity between energy donors and acceptors (18).

The next step in our evaluation was to determine the reporter fluorochrome compatibility. We found that VIC was not compatible with HEX and that Texas Red was not compatible with Alexa Fluor 594, while the rest of the fluorochromes were compatible (Table 2). From the data in Table 2, we selected 6-FAM, TET, VIC, and Alexa

Fluor 594 for further evaluation in the multiplex-QEXT assay (see the supplementary material and Supplementary Figure S3).

Finally, we optimized the 6-FAM, TET, VIC, and Alexa Fluor 594 probe concentrations. We evaluated concentrations in the range of 50 to 200 nM. The aim was to minimize the effect of crosstalk between VIC and TET. This was done by including a higher initial fluorescence of TET than VIC, since VIC gives crosstalk with TET, while TET does not crosstalk with VIC (see the supplementary material and Supplementary Figure S3). The optimized reaction contained 50 nM TET-labeled InIA1, 100 nM VIC-labeled InIA2, 200 nM 6-FAM-labeled InIA4, and 200 nM Alexa Fluor 594-labeled InIA3. An example of multi-component data for an InIA1 (TET) positive sample with the optimized probe concentrations is shown in

the supplementary material (see Supplementary Figure S3C).

Discrimination Between Target and Nontarget SNP Alleles

The change in fluorescence per labeling cycle follows an exponential decay function. The Delta outcome parameters in our macro (see the supplementary material) were determined by estimating the differences in reporter fluorescence before labeling and after complete probe labeling. Approximate values for these estimates were 1000, 700, 650, and 150 relative fluorescence units (rfu) for 50 nM TET-labeled InIA1, 100 nM VIC-labeled InIA2, 200 nM 6-FAM-labeled InIA4, and 200 nM Alexa Fluor 594-labeled InIA3, respectively.

We evaluated the incorporation rates for the different probe/fluorescence combinations using approximately 75 ± 25 nM template (quantified as described in the Materials and Methods section) with known SNP allele patterns (Figure 2). Generally, probe fractions that were labeled per cycle were between 3% and 6% for the target SNP alleles, while the fraction for the nontarget SNP alleles were below 1%. Furthermore, all the probe/fluorescence combinations gave highly significant discrimination between target and nontarget SNP alleles (*P* < 0.0005).

There was a linear response between the incorporation rate and the relative amount of the different SNP alleles in the mixed samples (Figure 3). The

Table 2. Compatibility in Multiplex Assays

Reporter	6-FAM	HEX	TET	VIC	Texas Red
HEX	++				
TET	++	+			
VIC	++	-	+		
Texas Red	++	++	++	++	
Alexa Fluor 594	++	++	++	++	-

Empiric evaluation based on signal differentiation between pairs of channels was determined by Sequence Detection System (SDS) 2.2 multicomponent analyses. ++, no interference between channels; +, slight interference between channels; -, signals could not be separated.

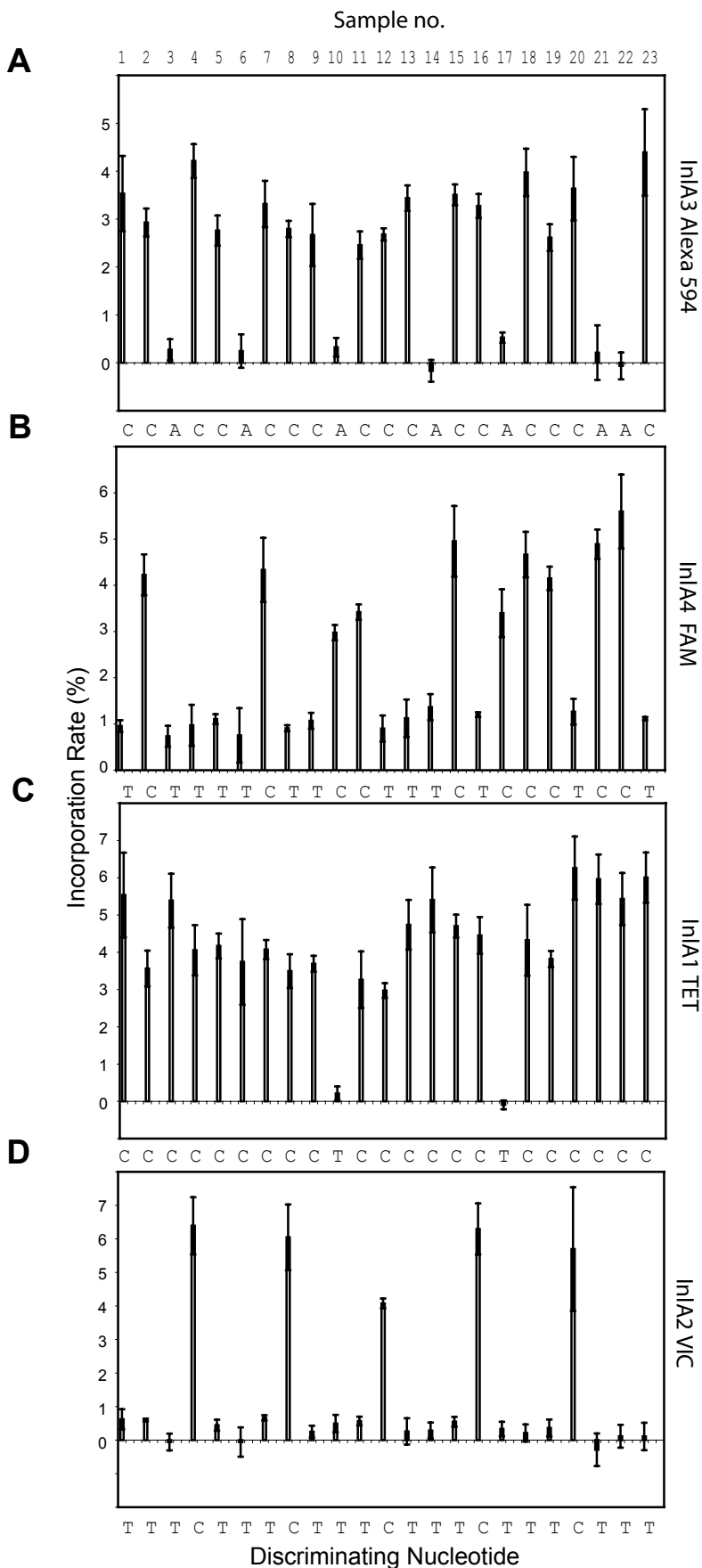


Figure 2. Four-plex single nucleotide polymorphism (SNP) scoring for pure isolates. Twenty-three strains with known *InIA* sequences were analyzed by multiplex quencher extension (multiplex-QEXT). The discriminating nucleotides are shown for each panel. Each panel represents the results for the different primer/probe combinations. (A) Probe InIA3 labeled with Alexa Fluor 594. (B) Probe InIA4 labeled with FAM. (C) Probe InIA1 labeled with TET. (D) Probe InIA2 labeled with VIC. The following strains were analyzed: 1, SS 12067; 2, NVH 762; 3, NVH 3035; 4, NVH 2114; 5, SS 9618; 6, NVH 3591; 7, NVH 921; 8, NVH 2010; 9, SS 8819; 10, NVH 1348; 11, NVH 2778; 12, NVH 1419; 13, SS 7751; 14, NVH 3477; 15, NVH 1037; 16, NVH 2014; 17, 3140; 18, SS 7785; 19, NVH 701; 20, NVH 39; 21, SS 65500; 22, NVH 502; 23, NVH 688. Error bars represent standard deviations for three independent replicates.

relative amounts of target SNP alleles of 10% or higher could be detected for all the probes ($P < 0.05$), while the VIC-labeled InIA2 probe also enabled separate detection of samples containing 5% of the target ($P < 0.05$). Finally, we determined SNP allele frequencies in sample pools of strains ($n = 28$) with known SNP allele patterns. The mixing was based on the PCR product quantification described in the Materials and Methods section. We obtained a relatively good prediction of the SNP allele frequencies in these sample pools (Figure 3). Multiplex-QEXT is therefore promising for future adaptation to large-scale frequency screenings. SNP allele frequencies can give important information about the overall composition of *L. monocytogenes* populations, although individual SNP allele combinations (SNP haplotypes) cannot be determined from sample pools.

Screening of *L. monocytogenes* Diversity by Multiplex-QEXT

The frequency of the $2^4 = 16$ possible SNP allele combinations were evaluated among 252 *L. monocytogenes* strains (see Supplementary Table S1). We identified 12 SNP haplotypes, with seven of these accounting for more than 90% of the *L. monocytogenes* strains (see Supplementary Figure S4). There was a good correlation between serotyping and multiple-QEXT typing. Twenty-nine (88%) of the 33 strains confirmed as serotype 4 were within SNP allele group 4 (see Supplementary Figure S4). This SNP allele group also includes all three human epidemic strains. On the contrary, only two (1.5%) out of the 131 strains with serotype 1 were identified within SNP allele group 4.

Serotype 1 strains were present in 10 out of the 12 SNP groups identified, suggesting that this serotype does not represent a genetically distinct group. The SNP allele typing gave clearly better resolution than serotyping for strains belonging to serotype 1. Further advantages of multiplex-QEXT are the robustness and the possibility of large-scale screenings, for instance, in narrowing the group of *L. monocytogenes*

genes containing potentially pathogenic strains (19).

Serotyping is based on agglutination of antisera specific for either surface (O) or flagella (H) antigens. There are currently 16 serotypes defined by 15 O and 5 H antigens. Thus, 20 independent reactions are needed in order to run a full serotyping scheme. Clearly, the labor and costs with serotyping far exceed the costs with multiplex-QEXT.

Future Development of Multiplex-QEXT

Multiplex-QEXT should be adaptable to nucleotides other than cytosine. The multiplexing level of QEXT can potentially be increased by FRET-based detection in the near infrared (NIR) spectrum using current commercially available technology. For instance, FRET detection is widely documented for the NIR dyes LC-Red 640 and LC-Red 705 (20). These dyes

were not included in our assay, because the ABI PRISM 7900 HT sequence detector does not have an NIR detector.

The current limiting factor for the multiplexing level of QEXT is overlapping fluorochrome emission spectra. With future developments of fluorochromes with narrow emission spectra and by the use of better curve resolution technology, we foresee a great potential for increasing both the

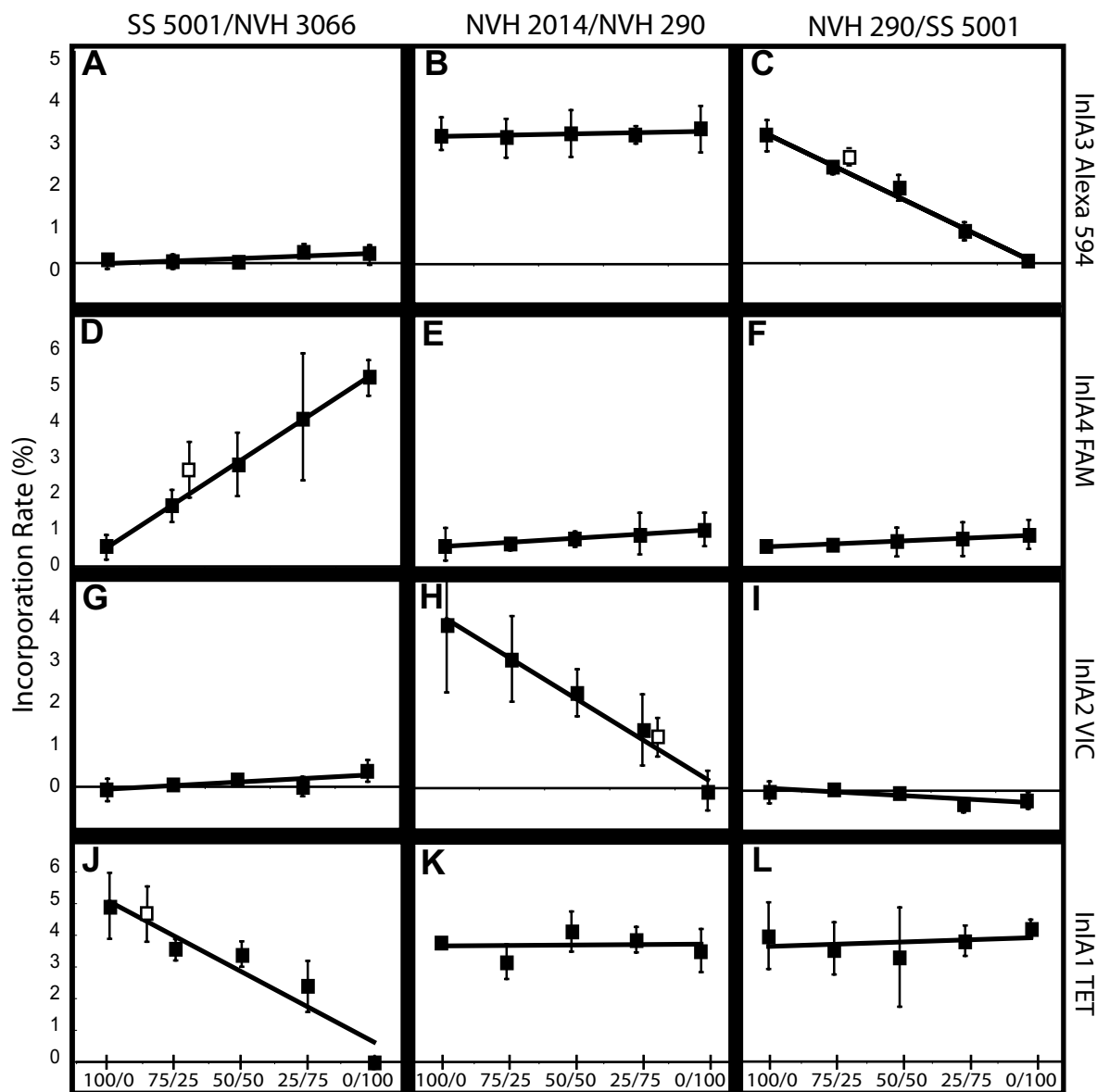


Figure 3. Quantitative evaluation of the four-plex quencher extension (QEXT) assay. Three series of mixed samples were analyzed. Each column represents series for the strains shown below the column with the mixing ratios (%) as indicated, while the rows represent the incorporation rates recorded for the different reporter signals. The average signals are shown as filled squares, with error bars showing standard deviations of three independent replicates. We also did similar experiments for pooled samples ($n = 28$), and the results are shown as open squares in panels C, D, H, and J, respectively.

multiplex level and the field of application of multiplex-QEXT.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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