E. COLI VTX1 AND VTX2 SUBTYPING PCR KIT

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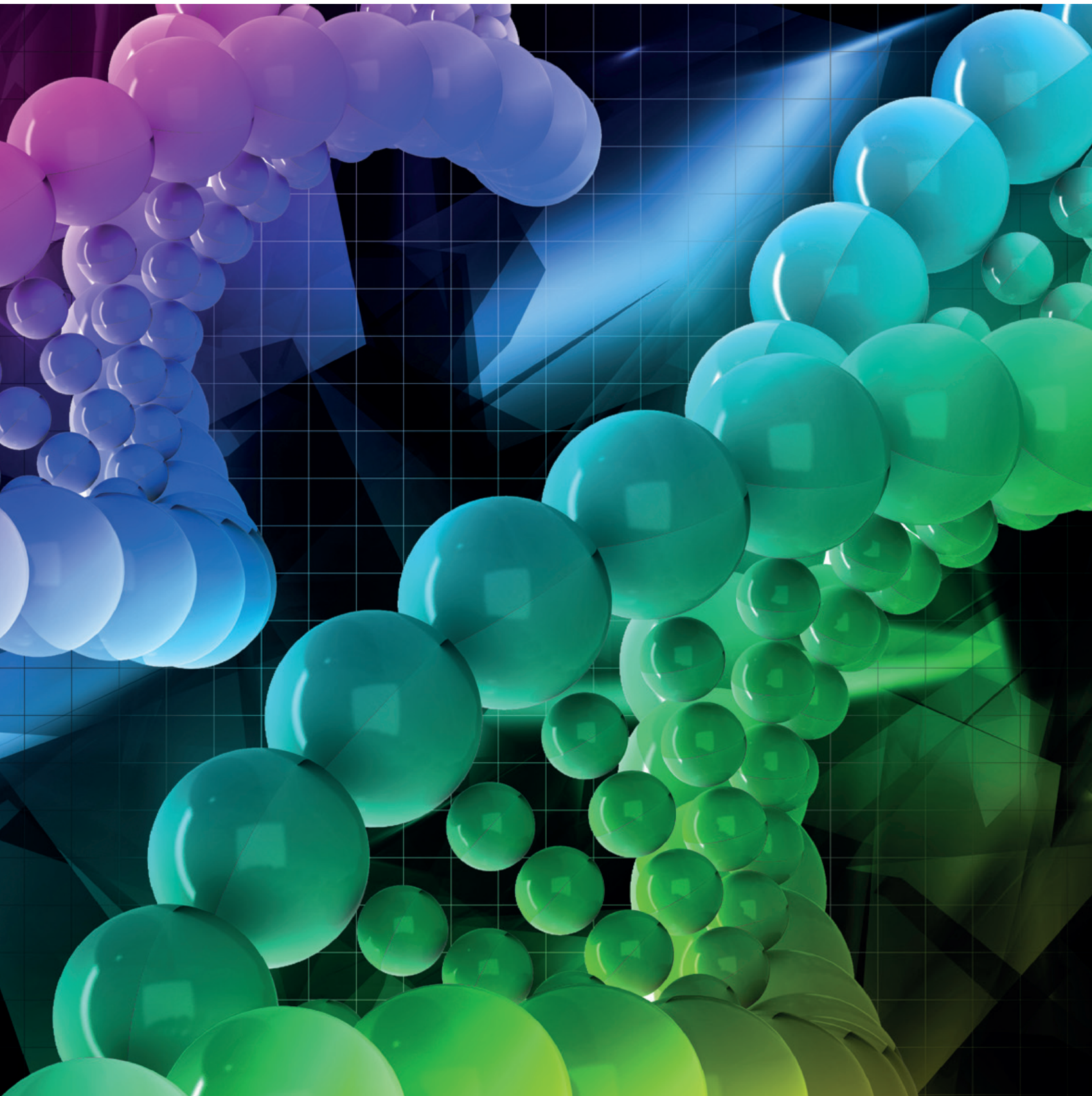


E. coli vtx1 and *vtx2* Subtyping PCR Kit

PCR Kit for detection of subtypes of the verocytotoxin encoding *E. coli* genes *vtx1* and *vtx2*

For *in vitro* diagnostic use only

Application

The *E. coli vtx1* and *vtx2* Subtyping PCR Kit is targeted for *in vitro* diagnostic PCR subtyping of the verocytotoxin encoding *E. coli* genes *vtx1* and *vtx2*.[1] In addition the ***vtx/eae* Detection** primer mix detects if the *vtx* type combines with the *eae* gene encoding intimin.

Description

The *E. coli vtx1* and *vtx2* subtyping PCR Kit contains seven primer mixes, seven PCR positive DNA controls, PCR ReadyMix (including loading buffer), TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and 10% Chelex-100 in TE-buffer (magnetic stir bar added). In addition, a test panel of two samples is included in order to test the temperature calibration of the in-house thermal cycler (see pages 6-8 for description of the test).

Table 1 shows the primer mixes and the appertaining control DNA samples, and lists the genes detected in the different primer mixes. Table 2 shows the amplicon size of all genes detected by this kit.

The kit contains enough PCR ReadyMix to perform approximately 200 PCR reactions. Primer mix ***vtx/eae* Detection** is intended for screening, and the vial contains reagent enough for 100 reactions. Primer mixes **vtx1 Subtyping**, *vtx2a/e* Subtyping, *vtx2c* Subtyping and *vtx2d* Subtyping contain reagents enough for 70 reactions each, whereas primer mix *vtx2b/f* Subtyping and ***vtx2g* Subtyping** contain reagents enough for 45 reactions. The volumes of the primer mixes are adjusted according to the frequency of subtypes found. Each control DNA vial contains 150 µL, corresponding to at least 25 tests.

Table 1. The seven primer mixes and the appertaining positive PCR controls are listed below:

Primer mixes and PCR positive controls	**Indentification of the genes**
Primer mix 1: *vtx/eae* Detection Control DNA 1: *vtx1*, *vtx2*, *eae*	*vtx1*, vtx2, *eae* and *16S* rDNA
Primer mix 2: *vtx1* Subtyping Control DNA 2: *vtx1a*, *vtx1c*, *vtx1d*	*vtx1a*, *vtx1c*, *vtx1d* and *16S* rDNA
Primer mix 3: *vtx2a/e* Subtyping Control DNA 3: *vtx2a*, *vtx2e*	*vtx2a*, *vtx2e* and *16S* rDNA
Primer mix 4: *vtx2b/f* Subtyping Control DNA 4: *vtx2b*, *vtx2f*	*vtx2b*, *vtx2f* and *16S* rDNA
Primer mix 5: *vtx2c* Subtyping Control DNA 5: *vtx2c* Optional sample D2432: *vtx2d* (as negative control)	*vtx2c* and *16S* rDNA
Primer mix 6: *vtx2d* Subtyping Control DNA 6: *vtx2d*	*vtx2d* and *16S* rDNA
Primer mix 7: *vtx2g* Subtyping Control DNA 7: *vtx2g*	*vtx2g* and *16S* rDNA

Table 2: Primer mixes included in the kit, genes detected and size of the amplicons

Primer mix	Genes	Amplicon size (bp)
vtx/eae Detection	*16s* rDNA	1062
	vtx2	625/627
	eae	377
	vtx1	209
vtx1 Subtyping	*16S* rDNA	1062
	vtx1a	478
	vtx1c	409
	vtx1d	203
vtx2a/e Subtyping	*16S* rDNA	1062
	vtx2e	592
	vtx2a	347/349
vtx2b/f Subtyping	*16S* rDNA	1062
	vtx2f	465
	vtx2b	251
vtx2c Subtyping	*16S* rDNA	1062
	vtx2c	177
vtx2d Subtyping	*16S* rDNA	1062
	vtx2d	280 235 (variant vtx2d-O55-5905) 179
vtx2g Subtyping	*16S* rDNA	1062
	vtx2g	573

All primers are synthetic single-stranded oligonucleotides with free 5'- and 3'- hydroxyl ends. Primer concentrations have been adjusted in all mixes for optimal performance in the multiplex PCRs. The PCR positive controls consist of purified DNA from *E. coli* strains containing the *vtx1* and/or the *vtx2* subtype genes. In some cases the strains contain the *eae* gene as well.

Materials Required but not Provided

Agar plates
2% agarose gels
100 bp DNA marker
Tubes for template preparation
Tube cap locks

The *E. coli* vtx1 and *vtx2* Subtyping PCR Kit has been tested with the thermal cycler Veriti and AB9700 from Applied Biosystems and S1000 from BioRad.

Important note: As this PCR Kit detects **subtypes** of the *vtx1* and *vtx2* genes, the temperature calibration of the thermal cycler is very important. Small differences in temperature and temperature fluctuations in the reaction block may result in ghost bands and thus in misinterpretations of the PCR results. In order to avoid misinterpretations, we have included a test panel of two samples in the kit (D2587 and D2432). A simple PCR set-up, analysing the two different templates with one primer mix (*vtx2c* Subtyping) at two different annealing temperatures (65°C and 66°C), determines whether the annealing temperature for the specific thermal cycler has to be increased compared to the described protocol. It is recommended to perform this test before the *vtx* subtyping is initiated (see procedure below).

Testing the vtx Subtyping System on the In-house Thermal Cycler

Procedure

Step 1

If the in-house thermal cycler has a single reaction module, prepare total master mix for three samples using the primer mix *vtx2c* Subtyping (see item 1 page 10). Dispense mixture and template in three PCR tubes as stated below:

Tube 1: 16 µL mastermix + 4 µL TE buffer
Tube 2: 16 µL mastermix + 4 µL D2587 (vtx2c positive)
Tube 3: 16 µL mastermix + 4 µL D2432 (vtx2d positive)

This gives the first set of samples with a total volume of 20 µL/reaction.

Step 2

Run the first set of samples under the PCR conditions described under item 3 (page 10) but with an annealing temperature at 65°C.

Step 3

After ending the first run, repeat step 1 to prepare the second set of samples. Run the samples under the same conditions but with an annealing temperature at 66°C.

If the in-house thermal cycler has a dual reaction module prepare both set of samples at once (total master mix for 6 samples) and run both sets simultaneously, but at the two different annealing temperatures (65 °C and 66 °C).

Step 4

Run 18 µL of each completed PCR reaction in separate wells on an 2% agarose gel.

Interpretation of the analysis results:

Compare the results obtained from the runs at the two different thermal cycler conditions. Primer mix ***vtx2c* Subtyping** is advised to be run at an annealing temperature of 66°C (see item 3 on page 10).
If the PCR reaction is performed at an annealing temperature of 65°C, it will most likely result in amplification of a strong *vtx2c* fragment at 177 bp analysing template D2587 (because D2587 is *vtx2c* positive), and a ghost band at 177 bp analysing D2432, (a false positive result as D2432 is *vtx2d* positive) (see figure 1). If the PCR reaction is performed at an annealing temperature of 66°C, the amplified *vtx2c* fragment at 177 bp should still be strong analysing template D2587, but the ghost band observed using D2432 as template should be eliminated.

If this result is observed, use the advised annealing temperatures (64°C and 66°C) described in item 3 (page 10) when performing the *vtx* subtyping. If a ghost band at 177 bp is still visible running the PCR reaction with template D2432 at an annealing temperature of 66°C, increase the advised annealing temperature by one degree celcius (i.e. run the PCR reactions at 65°C and 67°C) when performing the *vtx* subtyping.

Testing the *vtx* subtyping system on the in-house thermal cycler using the primer mix *vtx2c* Subtypning

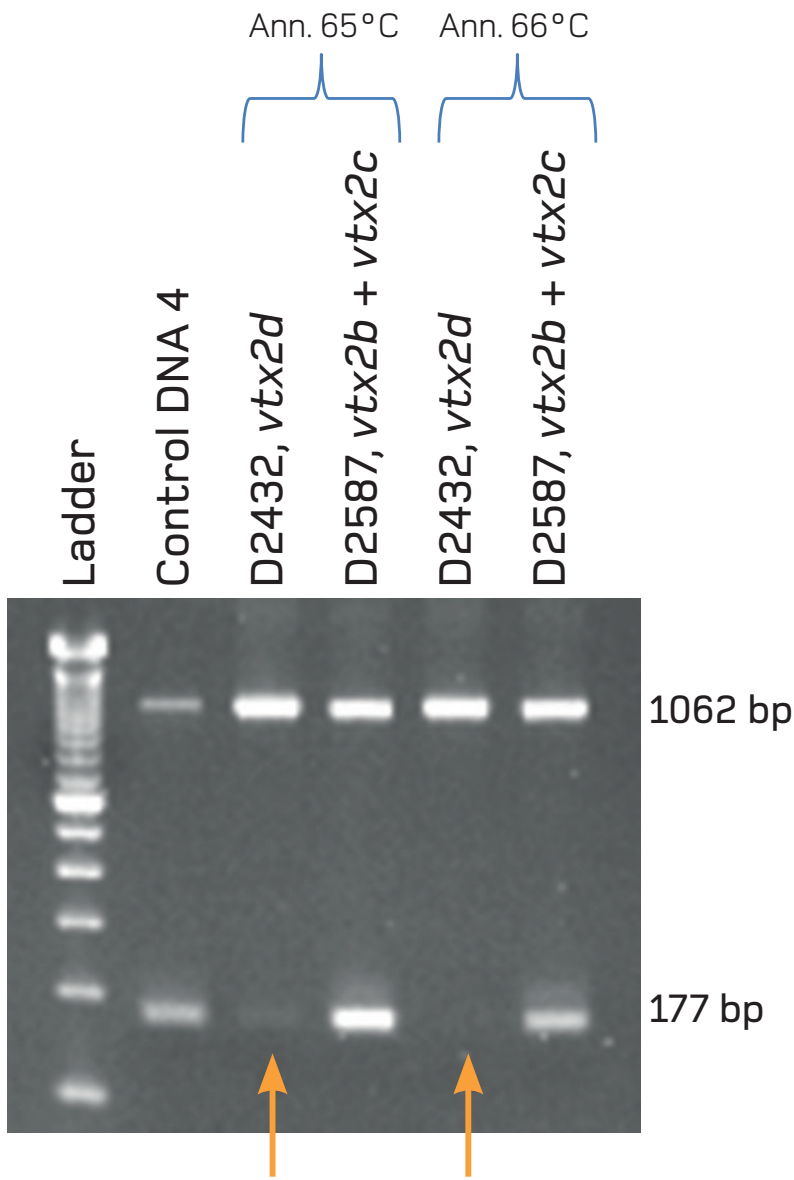


Figure 1. The ghost band amplified analysing the *vtx2d* strain (D2432) with primer mix *vtx2c* Subtyping disappears when the annealing temperature (ann.) is increased from 65°C to 66°C.

vtx Subtyping

Procedure

Template preparation

Template preparation and PCR setup should be performed in dedicated areas free of possible contamination.

1. Place the bottle with 10% Chelex-100 on a magnetic stirrer and pipette 200 µL/tube while the Chelex-100 is in solution. Pick up to 10 plate-grown colonies (if a pure *vtx* culture is being tested, only pick 3 colonies) and suspend them in the aliquoted Chelex-100 solution.
2. Boil the suspension for 5 min (remember tube cap locks) and centrifuge briefly (5 min at app. 2200 x *g*).
3. Dilute 15 µL of the supernatant in 100 µL TE-buffer and use 4.0 µL directly in the PCR.

PCR set-up

1. First prepare PCR reactions with the primer mix ***vtx/eae* Detection** to screen for *vtx/eae* positive samples. For faster subtyping, also prepare tests with primer mixes ***vtx1* Subtyping**, ***vtx2a/e* Subtyping**, ***vtx2b/f* Subtyping** and ***vtx2g* Subtyping** as all five PCR tests occur under the same thermal cycler conditions (annealing temperature at 64°C – see item 3 page 10). PCR reactions with primer mixes ***vtx2c* Subtyping** and ***vtx2d* Subtyping** are performed under the same thermal cycler conditions except for the annealing temperature which should be 66°C. If fast subtyping is needed, and if the thermal cycler equipment allows it, run both set-ups (all seven tests) simultaneously. Otherwise, set up the relevant PCR reactions for the vtx positive samples identified using the ***vtx/eae* Detection** primer mix.

Prepare the total master mix for each of the primer mixes to be run, and dispense 16 µL of the mixture in each PCR tube. Each tube has to contain the following amount of reagents:

Mastermix:	
PCR ReadyMix	10.0 µL
Primer mix	6.0 µL
Total	16.0 µL

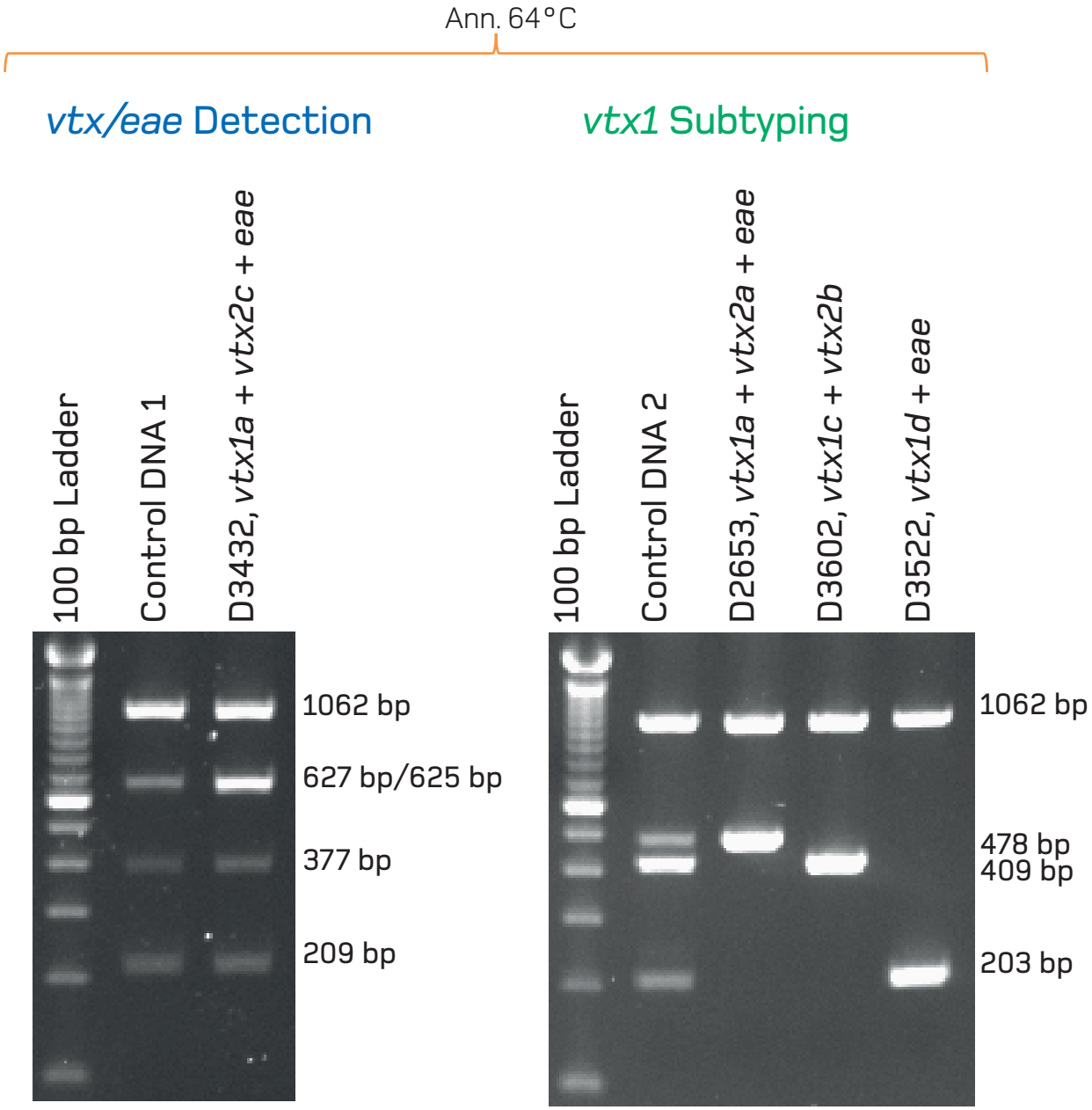
2. Add 4.0 µL template DNA (sample or positive control) to each tube and mix. Prepare a negative control by adding 4.0 µL TE-buffer to one of the tubes.
3. Run the PCR amplification in a thermal cycler under the conditions described below. Remember to choose the annealing temperature according to the specific primer mix:

Step	**Temperature**		**Time**
Initial denaturation	**95°C**		2 min
35 cycles of:			
Denaturation	**94°C**		50 sec
		N.B. different annealing temperatures.	
Annealing	**X**	**64°C** for *vtx/eae* Detection, *vtx1* Subtyping, *vtx2a/e* Subtyping, *vtx2b/f* Subtyping and *vtx2g* Subtyping.	40 sec
		66°C for *vtx2c* Subtyping and *vtx2d* Subtyping.	
Extension	**72°C**		50 sec
Final extension	**72°C**		3 min

4. Run 18 µL of each completed PCR reaction in separate wells on an agarose gel (2.0%) capable of separating the particular amplicon sizes (see table 2).

Interpretation of the Analysis Results

The figures below show results from PCR reactions using each of the seven primer mixes with control DNA and *vtx* positive samples. The fragments amplified are compared to a 100 bp DNA marker.

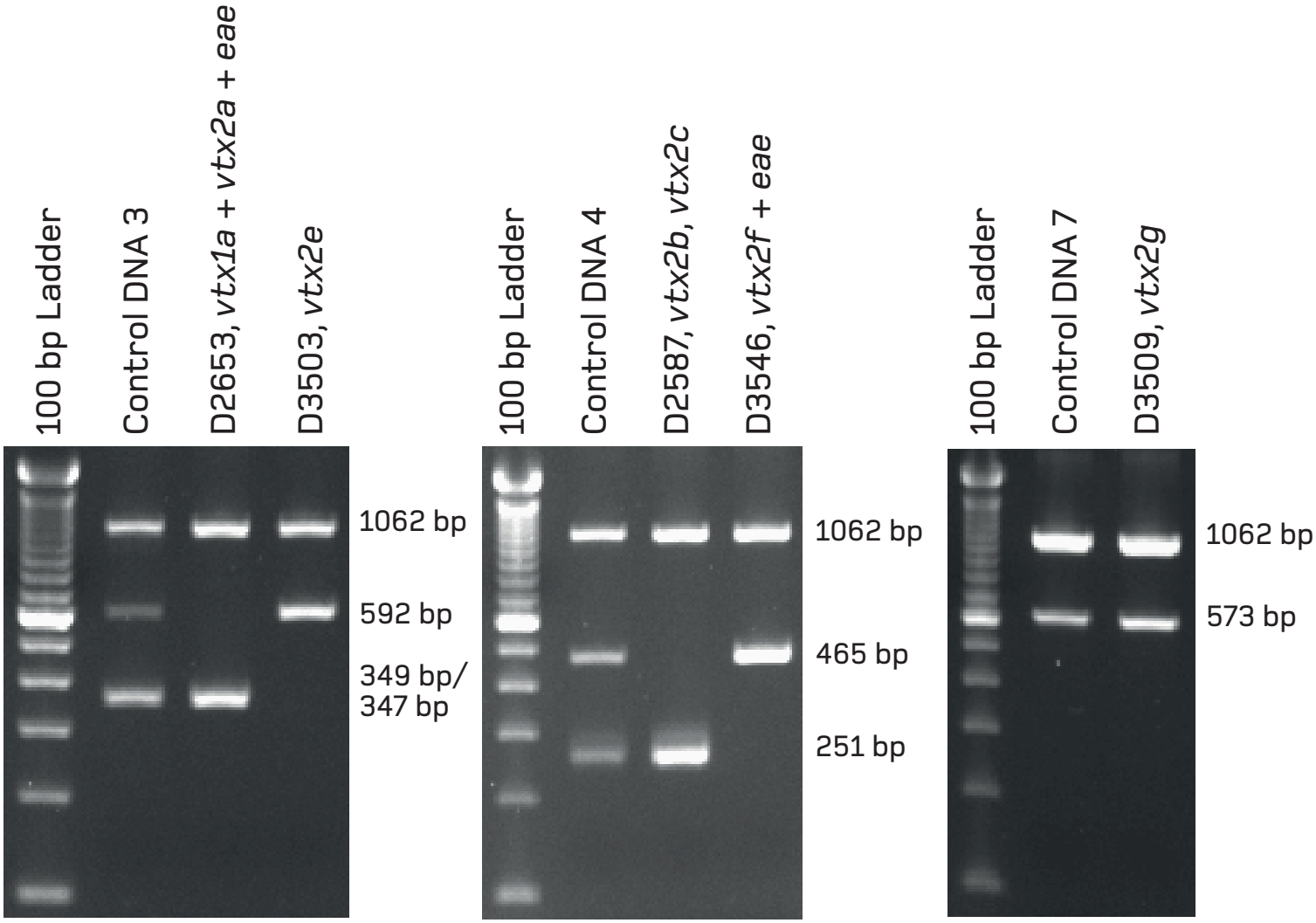


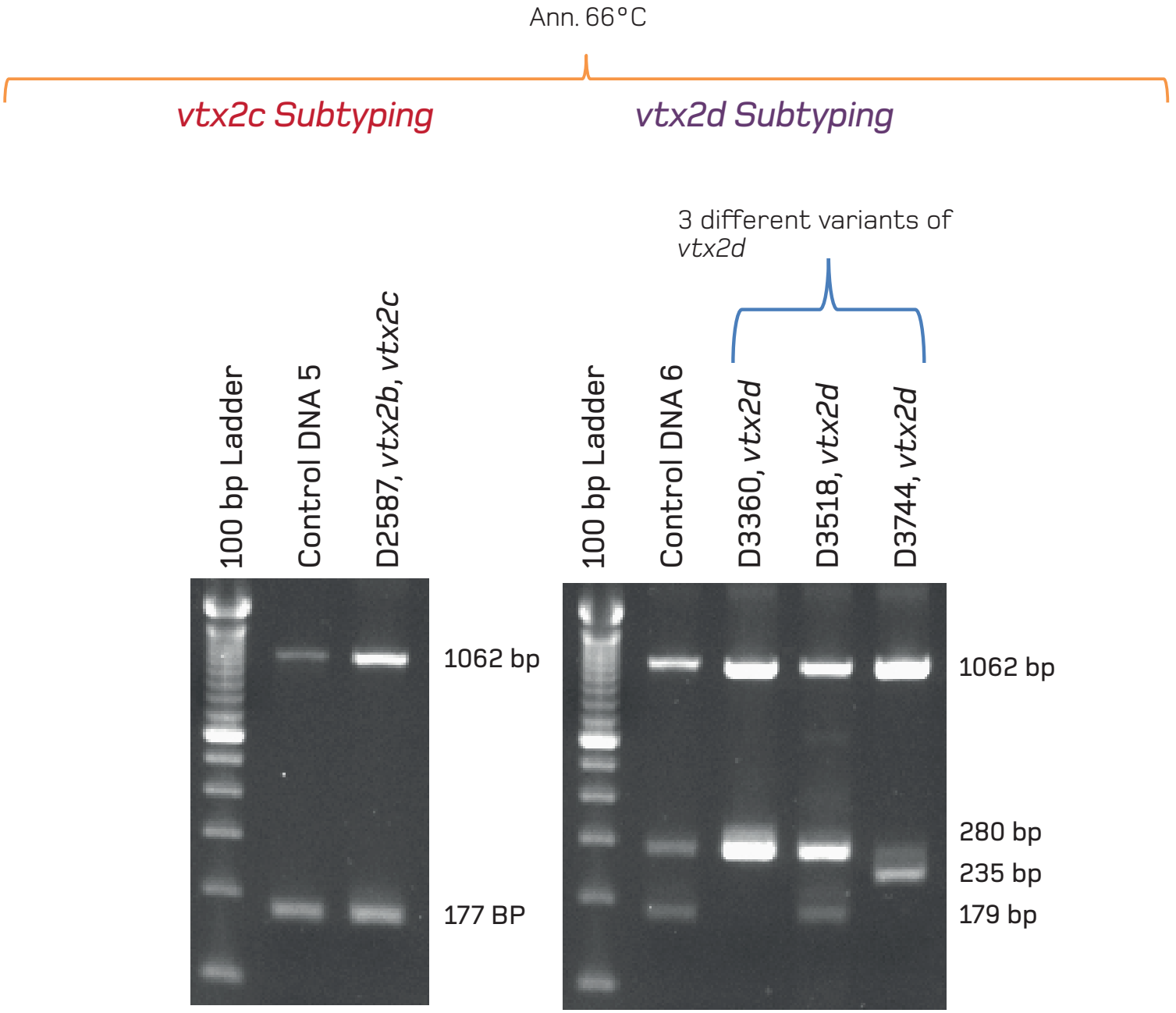
Ann. 64°C

vtx2a/e Subtyping

vtx2b/f Subtyping

vtx2g Subtyping





Recovery

Each of the 7 primer mixes have been tested with a panel of strains from "The International *Escherichia* and *Klebsiella* Centre (WHO)", Statens Serum Institut, Denmark. Primer mix ***vtx/eae* Detection** was tested with a panel of 115 strains which was a mix of *vtx1*, *vtx2*, and/or *eae* positive strains and non-virulent strains.

Primer mix *vtx1* Subtyping was tested with a panel of 61 *vtx1*, *vtx2*, and/or ***eae*** positive strains, and primer mix *vtx2a/e* Subtyping, *vtx2b/f* Subtyping, *vtx2c* Subtyping, *vtx2d* Subtyping, and ***vtx2g*** Subtyping were tested with a panel of 44 *vtx2* positive strains. Overall, the recovery (percentage of samples giving the expected result) was between 97 % and 100 % when compared to results obtained by doing PCR, sequencing, and/or DNA hybridisation technique at "The International *Escherichia* and *Klebsiella* Centre (WHO). The percentage of recovery for each primer mix is shown in the table below:

Primer mix	Recovery
vtx/eae Detection	97 %
vtx1 Subtyping	100 %
vtx2a/e Subtyping	98 %
vtx2b/f Subtyping	100 %
vtx2c Subtyping	98 %
vtx2d Subtyping	98 %
vtx2g Subtyping	100 %

Detection Limit

A pure *vtx2a* culture was serially diluted 10 times (resulting in 10^5-10^1 CFU/PCR) and mixed with a non-virulent *E. coli* strain to a final concentration of 10^4 CFU/PCR. A positive result was obtained with the primer mix *vtx2a/e* Subtyping until 1000 CFU/PCR. The same sensitivity was obtained when a *vtx2c* positive culture was tested together with a non-virulent *E. coli* strain and the primer mix *vtx2c* Subtyping.

Storage and Shelf Life

The PCR ReadyMix, the primer mixes, the DNA controls, and the two tubes containing template should be stored at -20°C. If necessary, dispense the reagents into several aliquots and store them at -20°C. The primer mixes and the controls in use can be stored at 4-8°C for up to 2 weeks. 10% Chelex-100 and TE-buffer should be stored at room temperature.

The expiry date of the kit is printed on the label.

Information and Ordering

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Quality System DS/EN ISO 13485

CE

References

1. Scheutz, F., Teel, L.D., Beutin, L., Piérard, D., Buvens, G., Karch, H., Mellmann, A., Caprioli, A., Tozzoli, R., Morabito, S., Strockbine, N.A., Melton-Ccelsa, A.R., Sanchez, M., Persson, S. and O'Brien, A.D. (2012): Multicenter Evaluation of a Sequence-Based Protocol for Subtyping Shiga Toxins and Standardizing Stx Nomenclature. J. Clin. Microbiology 50(9): 2951-2963.

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