For *in vitro* Veterinary Diagnostics only.

Kylt®

Kylt® APEC qPCR

Real-Time PCR Detection

www.kylt.eu

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Revision No.	Amendments
002	changed kit composition and reaction setup - the 4 different Positive Controls (APEC1, APEC2, APEC3, APEC4) have been replaced by one Positive Control combining all targets.

A. General

- Kylt® APEC qPCR kits are intended for the specific detection of bacterial DNA from 15 Avian Pathogenic *Escherichia coli* virulence factors in isolates derived from cultural processes with suitable sample material originating from birds. The following Avian Pathogenic *Escherichia coli* virulence factors are detected:

Group	Virulence factor	Description	Detected using Reaction-Mix:			
			APEC1	APEC2	APEC3	APEC4
Adhesins	F11	Serotype F11 of P fimbriae		x		
	papC	Pilus associated with pyelonephritis	x			
	tsh	Temperature-sensitive hemagglutinin	x			
Toxins	EAST1 (astA)	Heat-stable cytotoxin associated with enteroaggregative *E.coli*		x		
	vat	Vacuolating autotransporter toxin			x	
Invasins	ibeA	Invasion of brain endothelium			x	
Iron acquisition	iucD	Aerobactin synthesis		x		
	irp2	Iron-repressible protein (yersiniabactin synthesis)	x			
	iroN	Catecholate siderophore (salmochelin) receptor				x
	iutA	Aerobactin receptor			x	
	sitA	Iron transport system				x
Protectins/ Serum resistance	cvi/cva	Structural genes of colicin V operon (microcin ColV)			x	
	iss	Increased serum survival		x		
Miscellaneous	ompT	Outer membran protein, protease				x
	hlyF	Hemolysin				x

- The qualitative testing with Kylt® APEC qPCR kits is based on four multiplex Real-Time PCRs: In one reaction setting, 4 target genes encoding Avian Pathogenic *Escherichia coli* virulence factors are amplified in parallel by respective primer pairs in the Polymerase Chain Reaction (PCR). For Reaction-Mix APEC1 a target for the exogenous control (Internal Amplification Control (IAC)) is amplified in parallel with three target genes encoding Avian Pathogenic *Escherichia coli* virulence factors. Amplified target gene fragments are detected via fluorescently labeled probes during the PCR reaction in real-time (Real-Time PCR).
- The probes specific for detection of amplified target genes and the exogenous control are labeled with fluorescent dyes FAM, HEX, Cy5 and TXR, respectively, and their emitted fluorescence is separately optically measured by the Real-Time PCR thermal cycler. By means of all individual analyses per reaction vessel per sample and the Negative Control and Positive Control per run the APEC virulence factor-specific status of a sample can be evaluated in the end. This way, results can be achieved within a few hours after sample receipt.
- These kits were developed for use by trained laboratory personnel following standardized procedures. This Direction For Use must be followed strictly.

B. Reagents and Materials

- The following Kylt® APEC qPCR kits are available and comprise the following reagents:

Reagent	Colour of Lid	25 Reactions Article No 31746	Store at
Reaction-Mix (APEC1)	orange	1 x 450 µl	≤ -18 °C
Reaction-Mix (APEC2)	green	1 x 450 µl	≤ -18 °C
Reaction-Mix (APEC3)	brown	1 x 450 µl	≤ -18 °C
Reaction-Mix (APEC4)	yellow	1 x 450 µl	≤ -18 °C
Positive Control	red	4 x lyophilizate (final 50 µl each)	≤ -18 °C
Negative Control	blue	1 x 1 ml	≤ -18 °C

- After receipt, the components are immediately stored at ≤ -18 °C. Avoid repeated freezing and thawing of all the reagents and keep them thawed as short as possible. If occasional processing of few samples only is expected you may prepare appropriate aliquots of reagents before storage at ≤ -18 °C. Prepare aliquots in such a way that freeze-thaw-cycles are reduced to a maximum of three. The Negative Control can alternatively be stored at +2°C to +8°C.
- The components are to be used within the indicated shelf life (see box label). The components of different batches may not be mixed.
- The components are to be used within the indicated shelf life (see box label). The components of different batches may not be mixed.
- Before its first use, rehydrate the Positive Control: add 50 µl of Negative Control per vial, briefly incubate at room temperature and mix thoroughly by repeated vortexing. It is recommended to generate aliquots of suitable volumes and store them at ≤ -18 °C.
- The Reaction-Mix needs to be stored protected from abundant light. Do not expose to direct (sun)light.
  Attention: Each Reaction-Mix must be combined with the Positive Control (see chapter 3. " Reaction Setup and Amplification (Real-Time PCR)".)

C. Equipment and Reagents not included

- This detection method can be used on all commercially available Real-Time PCR thermal cyclers that detect the emitted fluorescence of the fluorescent dyes Cy5, FAM, TXR and HEX (emission 670, 520, 620 and 550 nm , respectively). Note that default normalization option against ROX (e.g. using ABI cyclers) must be deactivated.
- Apart from the disposables, the following further devices are needed and are not included in the Kylt® APEC qPCR kits:
  - DNA preparation kit / protocol (e.g. Kylt® RNA / DNA Purification products)
  - Table top microcentrifuge
  - Vortex
  - Micropipettes covering volumes of 1 µl to 1000 µl
  - Centrifuge for PCR tubes or plates
- Accessory Kylt® products: see chapter F "Related and Accessory Products".
- We recommend the exclusive use of certified Nuclease-free disposables as well as powder-free protective gloves. Please wear gloves during the entire experimental procedure. Gloves need to be changed frequently, especially after spillage or suspected contaminations.

D. Control Reactions

- The Positive Control allows for control of the specificity and efficiency of the reagents and the reaction itself, including the performance of the Real-Time PCR and of the Real-Time PCR thermal cycler.
- The Negative Control allows for exclusion of contaminations. The sample testing is only valid if both, Positive and Negative Controls, are used and verified for validity in every Real-Time PCR run.
- The Internal Amplification Control is included in the Reaction-Mix in a defined copy number; it is co-amplified (channel HEX) with every single reaction to detect possible inhibitory effects of the DNA preparation on the Real-Time PCR itself and thus to verify true-negative results..
- If appropriate sampling is unsure we recommend to analyze the samples in parallel with Kylt® Host Cells Real-Time RT-PCR Detection for presence of amplifiable nucleic acids derived from host cell material, see chapter F "Related and Accessory Products".

E. Protocol *(see also „Protocol At A Glance" at the end of this Direction For Use)*

- The overall protocol of the analysis consists of the following main workflow:

1. Sample Preparation
2. DNA Preparation
3. Reaction Setup and Amplification (Real-Time PCR)
4. Data Analysis – Validity and Qualitative Result

- We recommend proceeding through the protocol without interruption to avoid potential degradation of the processed samples and reagents. If necessary, you may store the final DNA preparation at ≤ -18 °C until further processing. Avoid repeated freezing and thawing of the DNA preparations.

1. Sample Preparation

- Material derived from cultural processes, i.e. colony material, is directly transferred into respective tubes, such as conical screw cap tube; therefore a little amount of a single colony is picked with a sterile loop wire or sterile pipette tip and transferred to the tube.

2. DNA Preparation

a) Kylt® RNA/DNA Purification products

- All kinds of sample matrices, including pure isolates, swabs, tissues and organs may be processed with Kylt® RNA/DNA Purification products (please refer to chapter F "Related Products").
- For detailed information on the DNA preparation process, please refer to the respective Direction For Use.

b) Alternative methods

- All kinds of sample matrices, including pure isolates, swabs, tissues and organs may be processed with appropriate DNA preparation kits or appropriate in-house methods.
- For detailed information on the DNA preparation process, please refer to the Direction For Use or Standard Operating Procedure of the specific kit or in-house method, respectively.

3. Reaction Setup and Amplification (Real-Time PCR)

- Before each use, briefly vortex and spin down the Reaction-Mix and Negative Control.
- To determine the total number of reactions needed per Reaction-Mix, count the number of samples and add 2 more for the Negative Control and the Positive Control. If one sample is to be analysed, 12 different reactions are required; an example of the reaction setup is given in the table below.

Min. Reaction Setup for Samples / Negative Control / Positive Control		
Reaction-Mix APEC1 + Sample 1	Reaction-Mix APEC1 + Negative Control	Reaction-Mix APEC1 + Positive Control
Reaction-Mix APEC2 + Sample 1	Reaction-Mix APEC2 + Negative Control	Reaction-Mix APEC2 + Positive Control
Reaction-Mix APEC3 + Sample 1	Reaction-Mix APEC3 + Negative Control	Reaction-Mix APEC3 + Positive Control
Reaction-Mix APEC4 + Sample 1	Reaction-Mix APEC4 + Negative Control	Reaction-Mix APEC4 + Positive Control

- The Reaction-Mix is ready-to-use, add 16 µl to each of the PCR tubes or plate wells ("cavities").
- Keep exposure of the Reaction-Mix to (sun)light as short as possible and return it back to appropriate storage temperature right after application. Avoid the formation of bubbles when pipetting samples and controls.
- Add 4 µl of the Negative Control to the corresponding cavities and seal it individually, if possible.
- Add 4 µl of each DNA preparation to the corresponding cavities and seal them individually, if possible.
- To minimize risk of potential cross-contaminations, 4 µl of the Positive Control are added to the corresponding cavities after all previous samples and control reactions are set up. Before each use, briefly vortex and spin down the rehydrated Positive Control (see also chapter B "Reagents and Materials").

- If not already done, finally seal the cavities. It is recommended to briefly spin them down before the start of the Real-Time PCR run.
- Place the cavities in the Real-Time PCR thermal cycler and run the test with Kylt® Profile II as given below.

Kylt® Profile II				
Step No	**Description**	**Temperature**	**Duration**	
1	Activation of Polymerase	95 °C	10 min	
2	Denaturation	95 °C	15 sec	42 cycles
3	Annealing & Extension	60 °C	1 min	
4	Fluorescence Detection	channels FAM, HEX, Cy5 and TXR		

- Kylt® Profile II allows for combined run of this and most other Kylt® qPCR detection methods.
- Alternatively, the Kylt® Profile I given below can be applied. Kylt® Profile I allows for combined run of this and most other Kylt® qPCR detection methods as well as Kylt® RT-qPCR detection products that need Reverse Transcription, such as those for detection of viral RNA.

Kylt® Profile I				
Step No	**Description**	**Temperature**	**Duration**	
1	Reverse Transcription	50 °C	10 min	
2	Activation of Polymerase	95 °C	1 min	
3	Denaturation	95 °C	10 sec	42 cycles
4	Annealing & Extension	60 °C	1 min	
5	Fluorescence Detection	channels FAM, HEX, Cy5 and TXR		

- In the event of a combined Real-Time (RT-)PCR run, make sure all necessary channels are detected.
- Please follow the specified instructions of your Real-Time PCR thermal cycler as recommended by the manufacturer.

4. Data Analysis – Validity and Qualitative Result

General

- The amplification data can be processed automatically using the specific software tool of your Real-Time PCR thermal cycler. Alternatively, the threshold can be set manually considering the following directions: The threshold should cross the FAM-, HEX-, Cy5- and the TXR-curves in the linear increase of their slope (log scaling of the y-axis). By setting the threshold, the crossing points with the FAM-, HEX-, Cy5- and the TXR-curves determine the respective cycle threshold (Ct), which is negatively correlated with the initial concentration of copies of the target genes in the Real-Time PCR reaction.
- Only curves with the typical exponential amplification, meaning the curve of the raw data shows a flat baseline at the beginning, followed by a clear (exponential) slope in fluorescence and possibly reaching a plateau-phase (y-axis in log scaling), should be regarded as positive.
- The actual test analysis starts with the validity check of the entire Real-Time PCR run. Afterwards, by means of the Internal Control the validity of each sample reaction and its true test result can be verified according to the Ct-value of the Internal Control channel (HEX, Reaction-Mix APEC1). Finally, the APEC virulence factor-specific status of each sample is analyzed in all four reaction settings (FAM, HEX, Cy5 and TXR).
- Convenient and reliable sample data entry, Real-Time PCR start, final qualitative analysis and documentation can be conducted with the Kylt® Software, please inquire.

Test Evaluation - Reaction-Mix APEC1

Control Reactions

- The **Real-Time PCR test run for APEC1** is only **valid** if the curves of the control reactions can be evaluated as follows:

Control Reactions	Channel (target)			
	HEX Internal Control (IAC)	FAM (papC)	Cy5 (tsh)	TXR (irp2)
Negative Control	**positive**	negative	negative	negative
Positive Control	**positive**	**positive**	**positive**	**positive**

- For a valid test the FAM-, Cy5- and TXR-Ct-value of the Positive Control has to be > 15 and ≤ 35. The HEX-Ct-values of the Positive and Negtive Control have to be ≤ 40.

Samples

Target	Channel	Signal				
Internal Control (IAC)	HEX	**positive**	positive / negative	positive / negative	positive / negative	negative
papC	FAM	negative	**positive**	negative	negative	negative
tsh	Cy5	negative	negative	**positive**	negative	negative
irp2	TXR	negative	negative	negative	**positive**	negative
The sample is		**papC, tsh and irp2 negative**	**papC positive**	**tsh positive**	**irp2 positive**	**inhibited**

- A **sample** is **negative for virulence factors papC, tsh and irp2,** if its HEX-curve is positive (Ct ≤ 40), but its FAM-, Cy5- and TXR-curves are negative.
- A **sample** is **positive for virulence factor papC,** if its FAM-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor tsh,** if its Cy5-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor irp2,** if its TXR-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **inhibited** if neither the HEX-curve nor the FAM-, Cy5- or TXR-curve are positive.
- For this reaction **cut-off values have to be set** for the papC, tsh and irp2 specific channels. Only results of Ct-value below Ct 30 for the specific channels FAM, Cy5 and TXR are to be considered as valid and positive.
- The Ct cut-off has no impact on the sensitivity because the sample material for the Kylt® APEC Real-Time PCR Detection is colony material derived from cultural processes and therefore gives strong positive signals.
- **Recommendation:** In the case of an inhibited sample the test may be repeated with a dilution of the DNA preparation at e.g. 1:10 (9 volumes Negative Control + 1 volume DNA Extract or eluted DNA). The Negative Control is used as the diluting agent. Preferably, the entire DNA preparation process is repeated using Kylt® RNA/DNA Purification products or appropriate alternative.

Test Evaluation - Reaction-Mix APEC2

Control Reactions

- The **Real-Time PCR test run for APEC2** is only **valid** if the curves of the control reactions can be evaluated as follows:

Control Reactions	Channel (target)			
	HEX (iss)	FAM (iucD)	Cy5 F(11)	TXR (asta)
Negative Control	negative	negative	negative	negative
Positive Control	**positive**	**positive**	**positive**	**positive**

- For a valid test the Ct-values of the Positive Control has to be > 15 and ≤ 35.

Samples

Target	Channel	Signal				
iss	HEX	**positive**	negative	negative	negative	negative
iucD	FAM	negative	**positive**	negative	negative	negative
F11	Cy5	negative	negative	**positive**	negative	negative
asta	TXR	negative	negative	negative	**positive**	negative
The sample is		**iss positive**	**iucD positive**	**F11 positive**	**asta positive**	**APEC virulence factors iss, iucD, F11 and asta not detectable**

- A **sample** is **positive for virulence factor iss,** if its HEX-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor iucD,** if its FAM-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor F11,** if its Cy5-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor asta,** if its TXR-curve is positive (Ct ≤ 30), independent of the other curves.
- **APEC virulence factors iss, iucD, F11 and asta are not detectable,** if neither the HEX-curve nor the FAM-, Cy5- or TXR-curve are positive.
- A **sample** is **inhibited** if all four channels in this reaction are negative as well as the HEX-curve (internal control) in reaction setting 1 (with Reaction-Mix APEC 1, please also refer to page 8).
- For this reaction **cut-off values have to be set** for the iss, iucD, F11 and asta specific channels. Only results of Ct-value below Ct 30 for the specific channels FAM, Cy5 and TXR are to be considered as valid and positive.
- The Ct cut-off has no impact on the sensitivity because the sample material for the Kylt® APEC Real-Time PCR Detection is colony material derived from cultural processes and therefore gives strong positive signals.
- **Recommendation:** In the case of an inhibited sample the test may be repeated with a dilution of the DNA preparation at e.g. 1:10 (9 volumes Negative Control + 1 volume DNA Extract or eluted DNA). The Negative Control is used as the diluting agent. Preferably, the entire DNA preparation process is repeated using Kylt® RNA/DNA Purification products or appropriate alternative.

Test Evaluation - Reaction-Mix APEC3

Control Reactions

- The **Real-Time PCR test run for APEC3** is only **valid** if the curves of the control reactions can be evaluated as follows:

Control Reactions	Channel (target)			
	HEX (ibeA)	FAM (vat)	Cy5 (cvi/cva)	TXR (iutA)
Negative Control	negative	negative	negative	negative
Positive Control	**positive**	**positive**	**positive**	**positive**

- For a valid test the Ct-values of the Positive Control has to be > 15 and ≤ 35.

Samples

Target	Channel	Signal				
ibeA	HEX	**positive**	negative	negative	negative	negative
vat	FAM	negative	**positive**	negative	negative	negative
cvi/cva	Cy5	negative	negative	**positive**	negative	negative
iutA	TXR	negative	negative	negative	**positive**	negative
The sample is		**ibeA positive**	**vat positive**	**cvi/cva positive**	**iutA positive**	**APEC virulence factors ibeA, vat, cvi/cva and iutA not detectable**

- A **sample** is **positive for virulence factor ibeA,** if its HEX-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor vat,** if its FAM-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor cvi/cva,** if its Cy5-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor iutA,** if its TXR-curve is positive (Ct ≤ 30), independent of the other curves.
- **APEC virulence factors ibeA, vat, cvi/cva and iutA are not detectable,** if neither the HEX-curve nor the FAM-, Cy5- or TXR-curve are positive.
- A **sample** is **inhibited** if all four channels in this reaction are negative as well as the HEX-curve (internal control) in reaction setting 1 (with Reaction-Mix APEC 1, please also refer to page 8).
- For this reaction **cut-off values have to be set** for the ibeA, vat, cvi/cva and iutA specific channels. Only results of Ct-value below Ct 30 for the specific channels FAM, Cy5 and TXR are to be considered as valid and positive.
- The Ct cut-off has no impact on the sensitivity because the sample material for the Kylt® APEC Real-Time PCR Detection is colony material derived from cultural processes and therefore gives strong positive signals.
- **Recommendation:** In the case of an inhibited sample the test may be repeated with a dilution of the DNA preparation at e.g. 1:10 (9 volumes Negative Control + 1 volume DNA Extract or eluted DNA). The Negative Control is used as the diluting agent. Preferably, the entire DNA preparation process is repeated using Kylt® RNA/DNA Purification products or appropriate alternative.

Test Evaluation - Reaction-Mix APEC4

Control Reactions

- The **Real-Time PCR test run for APEC4** is only **valid** if the curves of the control reactions can be evaluated as follows:

Control Reactions	Channel (target)			
	HEX (iroN)	FAM (hlyF)	Cy5 (ompT)	TXR (sitA)
Negative Control	negative	negative	negative	negative
Positive Control	**positive**	**positive**	**positive**	**positive**

- For a valid test the Ct-values of the Positive Control has to be > 15 and ≤ 35.

Samples

Target	Channel	Signal				
iroN	HEX	**positive**	negative	negative	negative	negative
hlyF	FAM	negative	**positive**	negative	negative	negative
ompT	Cy5	negative	negative	**positive**	negative	negative
sitA	TXR	negative	negative	negative	**positive**	negative
The sample is		**iroN positive**	**hlyF positive**	**ompT positive**	**sitA positive**	**APEC virulence factors iroN, hlyF, ompT and sitA not detectable**

- A **sample** is **positive for virulence factor iroN,** if its HEX-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor hlyF,** if its FAM-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor ompT,** if its Cy5-curve is positive (Ct ≤ 30), independent of the other curves.
- A **sample** is **positive for virulence factor sitA,** if its TXR-curve is positive (Ct ≤ 30), independent of the other curves.
- **APEC virulence factors iroN, hlyF, ompT and sitA are not detectable,** if neither the HEX-curve nor the FAM-, Cy5- or TXR-curve are positive.
- A **sample** is **inhibited** if all four channels in this reaction are negative as well as the HEX-curve (internal control) in reaction setting 1 (with Reaction-Mix APEC 1, please also refer to page 8).
- For this reaction **cut-off values have to be set** for the iroN, hlyF, ompT and sitA specific channels. Only results of Ct-value below Ct 30 for the specific channels FAM, Cy5 and TXR are to be considered as valid and positive.
- The Ct cut-off has no impact on the sensitivity because the sample material for the Kylt® APEC Real-Time PCR Detection is colony material derived from cultural processes and therefore gives strong positive signals.
- **Recommendation:** In the case of an inhibited sample the test may be repeated with a dilution of the DNA preparation at e.g. 1:10 (9 volumes Negative Control + 1 volume DNA Extract or eluted DNA). The Negative Control is used as the diluting agent. Preferably, the entire DNA preparation process is repeated using Kylt® RNA/DNA Purification products or appropriate alternative.

F. Related and Accessory Products

Product	Article No	Reactions	Description
Kylt® RNA / DNA Purification	31315	50	Combined RNA and DNA purifi cation from veterinary samples (spin-column based).
Kylt® RNA / DNA Purification HTP	31826	4 x 96	Magnetic bead based combined RNA and DNA purification kit for veterinary diagnostic samples. Suitable for Kylt® Purifier and Kylt® Purifier 48.
Kylt® Purifier	31436	1 unit	Purification system for magnetic bead based kits. Up to 96 samples are processed in under 30 minutes. Intended for high-throughput laboratories.
Kylt® Purifier 48	31748	1 unit	Purification system for magnetic bead based kits. Up to 48 samples are processed in under 30 minutes. Intended for low to medium throughput laboratories.
Kylt® Purifier Spin Tips	31434	5 Sets	Plate with 96 separate spin tips, used by the Kylt® Purifer to mix the well contents by stirring. Sufficient for 480 samples.
Kylt® Purifier Plates	31435	20 Plates	Plates to be used for the several reactions and reagents during automated nucleic acid purification. Sufficient for 320 to 480 samples (depending on device and protocol) .

G. Ordering information

For a fast and efficient service please send your order to orders@kylt.eu and please provide the following information:

- Delivery address
- Invoice address
- Purchaser contact telephone number
- End user name and telephone number (if different)
- Purchase order number
- Product name and cataloge number
- Quantity and size of products
- Indicate if your account is VAT exempt

Production:

SAN Group Biotech Germany GmbH | Muehlenstr. 13 | 49685 Hoeltinghausen | Germany
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Development, manufacturing and distribution of Kylt® *In-Vitro* Diagnostics is certified to ISO 9001:2015.

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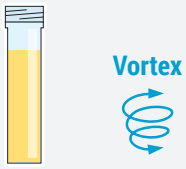
For veterinary use only. For *in vitro* use only. Regulatory requirements vary by country, not all of the products described herein may be available in your geographic area.

qPCR.APECqPCR.02, Rev002, April 2024

PROTOCOL AT A GLANCE
Real-Time PCR Setup

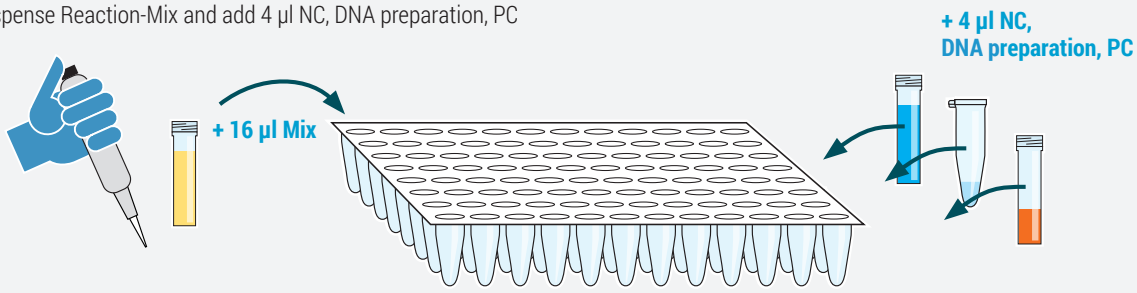
1

Pulse-vortex and spin down



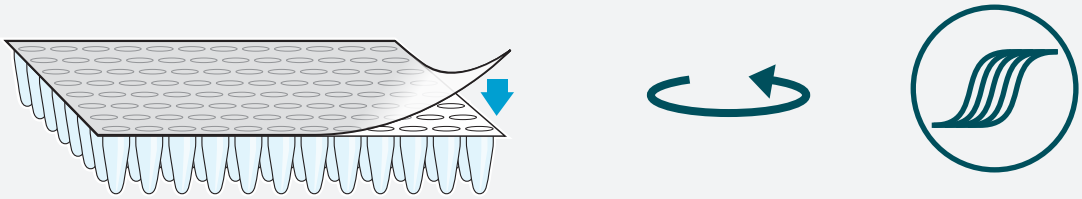
2

Dispense Reaction-Mix and add 4 µl NC, DNA preparation, PC



3

Seal cavities, spin down (recommended), and start cycler



4

Analysis