

Bladder EpiCheck® Kit

Instructions for Use *with ABI 7500 fast Dx*

Suitable for Bladder EpiCheck software version 1.9

Bladder EpiCheck DNA Extraction Kit	50 tests	P/N NX899090-01C
Bladder EpiCheck Test Kit	60 tests	P/N NX899090-02C

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1. Intended Use

The Bladder EpiCheck test is an in vitro diagnostic device for the detection of DNA methylation patterns in urine that are associated with urothelial carcinoma. It is intended for use as a noninvasive method for monitoring of tumor recurrence in conjunction with standard diagnostic procedures in patients previously diagnosed with bladder cancer and/or upper tract urothelial carcinoma. Additionally, Bladder EpiCheck is intended for use as an aid in the detection of bladder cancer and upper tract urothelial carcinoma (UTUC), in patients presenting with hematuria and/or other urinary tract symptoms and/or findings with a suspicion of malignancy, in conjunction with standard diagnostic procedures.

2. Summary and Explanation of the Test

Methylation aberrations are found in human cancers [1,2] and specifically in bladder cancer [3]. The Bladder EpiCheck kit is a real-time PCR assay, which detects aberrations in the methylation pattern of a panel of 15 DNA methylation biomarkers that were found to distinguish between patients with bladder cancer or upper tract urothelial carcinoma (UTUC) and patients without those cancers.

3. Principle of the Procedure

The Bladder EpiCheck kit analyzes the methylation pattern in a panel of 15 DNA methylation biomarkers and determines whether this pattern is consistent with a bladder cancer or UTUC positive or a bladder cancer and UTUC negative patient.

The procedure of the kit comprises of the following steps:

1. **Creating a cell pellet from the urine sample**
The urine sample is centrifuged such that the cells (both normal and cancerous, if present) are separated from the urine liquid.
2. **DNA extraction**
DNA is extracted from the cell pellet.
3. **Digestion of extracted DNA**
Extracted DNA is digested using a methylation-sensitive restriction enzyme, which cleaves DNA at its recognition sequence if it is unmethylated (methylated DNA remains intact).
4. **Real-time PCR amplification**
Real-time PCR amplification is performed in a 96-well plate using the Applied Biosystems 7500 Fast Dx instrument. Each EpiCheck run requires 2 control samples (Undigested Control and No Template Control) and therefore up to 10 patient samples can be analyzed. Each sample is analyzed in 8 wells corresponding to a single column of the

plate. In each well 3 biomarkers are amplified, except for mix F, in which 4 biomarkers are amplified. There are two types of biomarkers: control biomarkers and identification biomarkers. Control biomarkers (IR, DC, DCR) are used to verify that the sample was successfully processed. Identification biomarkers (BE-1 to BE-15) are used to determine whether a sample is bladder cancer or UTUC positive or negative. See [table 1](#) for a list of biomarkers per well and an explanation of their function.

5. Software analysis of the real-time PCR output file

The Bladder EpiCheck software analyzes the .sds file which is the output of the real-time PCR reaction. The software determines whether the EpiCheck run controls are OK, and if so, it proceeds to analyze the patient samples. For each sample (if its controls are OK) the software calculates its **EpiScore** – a number between 0 and 100 representing the overall methylation level of the sample at the panel biomarkers. If the EpiScore is equal or above 60 then the patient is defined to be Positive (indicating a high risk for bladder cancer or UTUC), otherwise the patient is defined to be Negative (indicating a low risk for bladder cancer or UTUC).

The principle components of the Test Kit are explained below.

PCR mixes A-H

The kit includes 8 PCR mixes designed to amplify the panel of 15 biomarkers as well as control biomarkers, as listed in the following [table 1](#):

Table 1 - PCR mixes

Mix	IR	Reporter
PCR mix A	IR	FAM
	BE-1	ROX
	BE-2	Cyanine5
PCR mix B	IR	FAM
	BE-3	ROX
	BE-4	Cyanine5
PCR mix C	IR	FAM
	BE-5	ROX
	BE-6	Cyanine5
PCR mix D	IR	FAM
	BE-7	ROX
	BE-8	Cyanine5
PCR mix E	IR	FAM
	BE-9	ROX
	BE-10	Cyanine5
PCR mix F	IR	FAM
	DC	JOE
	BE-11	ROX
	DCR	Cyanine5
PCR mix G	IR	FAM
	BE-12	ROX
	BE-13	Cyanine5
PCR mix H	IR	FAM
	BE-14	ROX
	BE-15	Cyanine5

IR (Internal Reference): a marker that does not contain a restriction site of the digestion enzyme, and therefore it is not affected by digestion. This marker is included in each well and serves as an internal control for the success of the amplification in that well, and specifically that there was a sufficient amount of DNA. If the amplification of IR fails in one or more of a sample's wells, analysis of the sample will not be performed and the software will provide an indication.

DC (Digestion Control) and **DCR** (Digestion Control Reference): non-genomic markers located on exogenous DNA sequences. After DNA extraction, the DC and DCR fragments are added to each DNA sample, and are used to verify that the digestion of the sample was successful. The DCR fragment does not contain a restriction site while the DC fragment contains a restriction site. If digestion is successful, the DCR marker is expected to be amplified successfully while the DC marker is expected to be digested and therefore not be amplified (or be amplified with low efficiency).

BE-1 to BE-15: The panel of 15 identification biomarkers used to determine whether a sample is bladder cancer or UTUC positive or bladder cancer or UTUC negative. Each marker contains a restriction site. Markers with a high level of methylation will have more intact template molecules following digestion, leading to a higher signal intensity in the PCR. Conversely, markers with a low level of methylation will have less intact template molecules following digestion, leading to a lower signal intensity in the PCR.

No Template Control (NTC)

Contains ultra-pure water (DNA free) to be used for the NTC reaction, which assesses potential contamination during assay setup.

Undigested Control (UC)

Contains 17 exogenous sequences representing the entire Bladder EpiCheck panel of biomarkers (except DCR which is added in the digestion step). These exogenous sequences are designed to be protected from restriction and therefore will result a high signal intensity at the PCR stage. The purpose of this sample is to verify that all primers and probes of the PCR mixes as well as the instrument detectors are working properly.

4. Performance Characteristics

4.1. Analytical Performance

Analytical Sensitivity – Limit of Detection (LoD)

LoD studies were performed for both the methylation percent and amount of DNA, based on guidance from the CLSI Standard: EP17-A2 (Evaluation of detection capability for clinical laboratory measurement procedures; approved guidelines – second edition). The LoD was established assuming that the limit of blank (LoB) is zero. The LoD was calculated by probit regression fitted curve with 95% confidence bounds.

Analytical sensitivity characteristics for the Bladder EpiCheck were observed as follows:

Table 2 - Analytical sensitivity characteristics for the Bladder EpiCheck

Performance Characteristic	Input DNA Concentration Range	Methylation Fraction
Limit of Detection	0.625 ng/well	0.2% of DNA molecules in a sample

Precision and Reproducibility

"Between lab" precision and reproducibility study was performed to assess potential variation of the Bladder EpiCheck assay based on guidance from the CLSI Standard: EP05-A3 (Evaluation of precision of quantitative measurement procedures; approved guidelines – third edition). As part of the study, a variance component analysis was performed by sample type (i.e. different EpiScore) for the Bladder EpiCheck assay to estimate the components of precision for each source of potential variation (operator, run, site, and replicate). The study was performed at two sites, with two operators at each site.

A total of 12 Bladder EpiCheck runs were performed at each site, 6 runs per operator. Each run included 4 human DNA samples with pre-defined EpiScores, including two replicates of each of the samples for a total of 192 measurements. Since the assay was performed three times over a period of 20 days the test was performed on human DNA samples as urine isn't stable for such a long time. Four human DNA sample types were used for each test (negative, high negative, low positive and high positive). The samples were supplied to the labs by Nucleix.

The repeatability and reproducibility (precision) and their 95% two-sided confidence intervals (CI) were calculated using a random effects analysis of variance model programmed in SAS® using the MIXED procedure. The confidence interval of the reproducibility was calculated with bootstrap methodology using 10,000 simulated samples. The overall agreement, sensitivity and specificity are presented by lab and across labs ([table 4 and 5](#)).

The mean EpiScores are presented in tabular form by sample type, along with their respective 95% confidence intervals (CI) (table 3). The binary responses by sample type are presented, by lab and across labs, and the overall, positive and negative agreement, as well as their sensitivity and specificity, along with their respective 95% confidence intervals (table 4 and 5).

Table 3 - Mean EpiScore – per sample type

Sample type	Mean	95% CI
Low Negative	11.2	[-6.5 - 28.9]
High Negative	44.3	[10.4 - 78.2]
Low Positive	73.2	[62.6 - 83.8]
High Positive	90.0	[82.3 - 97.7]

Table 4 - Binary response - per sample type and lab

Sample type	Lab1				Lab2				All			
	Negative		Positive		Negative		Positive		Negative		Positive	
	N	%	N	%	N	%	N	%	N	%	N	%
Low Negative	24	100	.	.	24	100	.	.	48	100	.	.
High Negative	22	91.67	2	8.33	24	100	.	.	46	95.83	2	4.17
Low Positive	.	.	24	100	.	.	24	100	.	.	48	100
High Positive	.	.	24	100	.	.	24	100	.	.	48	100

For the low negative, low positive and high positive samples, in both labs the agreement was 100% (48/48, 95% Wilson Score CI: [92.59%; 100.0%]). For the high negative sample, the agreement was 95.83% (46/48, 95% Wilson Score CI: [86.02%; 98.85%]). The overall agreement was 98.96% (190/192, 95% Wilson Score CI: [96.28%; 99.71%]). The overall sensitivity was 100% (96/96, 95% Wilson Score CI: [96.15%; 100.0%]). The overall specificity was 97.92% (94/96, 95% Wilson Score CI: [92.72; 99.43%]).

Table 5 - Binary response – Between Lab Agreement

Sample type	Result Lab 1	Result Lab 2			
		Negative		Positive	
		N	%	N	%
Low Negative	Negative	24	100	.	.
High Negative	Negative	22	91.67	.	.
	Positive	2	8.33	.	.
Low Positive	Positive	.	.	24	100
High Positive	Positive	.	.	24	100

For the low negative, low positive and high positive samples, the agreement as well as the average positive/negative agreement were 100% (24/24, 95% Wilson Score CI: [86.20%; 100.0%]). For the high negative sample, the agreement was 91.67% (22/24, 95% Wilson Score CI: [74.15%; 97.68%]) and the average negative agreement was 95.65% (22/((22+24)/2), 95% Wilson Score CI: [79.01%; 99.23%]).

Descriptive statistics were separately calculated for all sample combinations. Inter-lab descriptive statistics are provided below [\(table 6\)](#).

Table 6 - Simple Descriptive Statistics for EpiScore per Sample type – by Lab and Overall

Sample type	Lab	EpiScore					
		N	Mean	SD	Min	Median	Max
Low Negative	1	24	12.6	3.62	7	12	20
	2	24	9.8	3.09	6	9	19
	All	48	11.2	3.62	6	11	20
High Negative	1	24	47.0	9.04	32	45	72
	2	24	41.6	5.61	29	41	52
	All	48	44.3	7.91	29	44	72
Low Positive	1	24	74.0	1.62	71	74	77
	2	24	72.3	2.04	66	73	75
	All	48	73.2	2.00	66	73	77
High Positive	1	24	90.6	0.58	90	91	92
	2	24	89.4	0.78	88	90	91
	All	48	90.0	0.91	88	90	92

[Table 7](#) below presents the repeatability/reproducibility as observed in the study. With the Low Negative sample, the mean EpiScore was 11.2 and the repeatability 3.28 (CV: 29.3%). With the High Negative sample, the mean EpiScore was 44.3 and the repeatability 7.09 (CV: 16.0%). With the Low Positive sample, the mean EpiScore was 73.2 and the repeatability 1.80 (CV: 2.5%). With the High Positive sample, the mean EpiScore was 90.0 and the repeatability 0.68 (CV: 0.8%).

Table 7 - Repeatability and between labs, test days, operators and plates precision – per sample type

Sample type	Parameter	Value (SD)	95% CI	CV
Low negative	Repeatability	3.28	[2.70 - 4.17]	29.3%
	Between labs precision	3.73	[2.54 - 4.20]	33.3%
	Between test days precision	3.41	[2.48 - 4.09]	30.4%
	Between operators precision	3.28	[2.37 - 3.81]	29.3%
	Between plates precisions	3.28	[2.31 - 3.62]	29.3%
High negative	Repeatability	7.09	[5.82 - 9.07]	16.0%
	Between labs precision	7.59	[4.67 - 9.02]	17.1%
	Between test days precision	7.36	[5.00 - 9.17]	16.6%
	Between operators precision	7.56	[5.17 - 8.98]	17.1%
	Between plates precisions	7.09	[4.70 - 8.20]	16.0%
Low positive	Repeatability	1.80	[1.49 - 2.27]	2.5%
	Between labs precision	2.08	[1.24 - 2.63]	2.8%
	Between test days precision	1.80	[1.25 - 2.18]	2.5%
	Between operators precision	1.87	[1.24 - 2.37]	2.6%
	Between plates precisions	1.80	[1.17 - 2.13]	2.5%
High positive	Repeatability	0.68	[0.56 - 0.86]	0.8%
	Between labs precision	1.08	[0.77 - 1.28]	1.2%
	Between test days precision	0.68	[0.52 - 0.77]	0.8%
	Between operators precision	0.68	[0.53 - 0.78]	0.8%
	Between plates precisions	0.68	[0.50 - 0.80]	0.8%

In summary, the repeatability and between labs, test days, operators and runs precision results from this study are below the value of 20% of the mean for the high negative, low positive and high positive samples. In the low negative sample, despite the high variability, the binary result is still negative in all cases, thus this result has minimal impact on the reliability of the test.

Lot-to-lot reproducibility

Lot-to-Lot reproducibility was evaluated for the two components of the Bladder EpiCheck kit, the DNA Extraction Kit and the Test Kit, based on guidance from the CLSI Standards: EP05-A3 (Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline).

Lot-to-Lot reproducibility was assessed by testing a panel of samples comprised of four samples containing various levels of methylation. Three different lots of the Test Kit and three different lots of the DNA Extraction Kit were tested. This design yields a five way combination in which one lot of the Test Kit was tested with three different lots of the DNA Extraction Kit and one lot of the Extraction Kit was tested with three different lots of the test Kit.

The lot-to-lot experiment was performed on human urine samples spiked with human DNA samples with four different pre-defined methylation levels, (low negative, high negative, low positive and high positive). The samples were characterized as positive or negative based on the expected EpiScore.

Urine was collected from healthy donors, the urine was then centrifuged in order to separate the donor DNA from the urine. The DNA-free urine was split into four different tubes and then spiked with the human DNA samples under test.

Each of the five lot-to-lot combinations were repeated on 3 different days, with 2 replicates per run for each sample, for a total of 120 measurements

For each sample type, the EpiScore was modeled as a function of lot and day, entered as random effects. The mean measurement was estimated from the model of each sample type and is presented with a 95% confidence interval. Reproducibility was estimated from the model's variance components, with the overall value reported, as well as between lots and between days reproducibility. These are presented with 95% confidence intervals. The confidence intervals of the between lots and between days reproducibility (which are the sum of two variance components) were calculated with bootstrap methodology using 10,000 simulated samples. In addition, the measurements were categorized as positive or negative according to the Bladder EpiCheck algorithm threshold.

The overall agreement, sensitivity and specificity of the binary response are presented along with their respective 95% exact binomial confidence intervals ([table 8](#)).

Table 8 - Binary response - per sample type

Sample type	Results			
	Negative		Positive	
	N	%	N	%
Low Negative	30	100	.	.
High Negative	30	100	.	.
Low Positive	.	.	30	100
High Positive	.	.	30	100

For each sample type, the agreement in the binary response was 100.0% (30/30, 95% Wilson Binomial CI: [88.65%, 100.0%]). The overall agreement in the binary response was 100.0% (120/120, Wilson Binomial CI: [96.90%, 100.0%]). The overall sensitivity and specificity were 100.0% (60/60, Wilson Binomial CI: [93.98%, 100.0%]).

The between lots precision and reproducibility for both positive and negative samples were within the pre-specified acceptance criteria (<20%), ranging between 0.2% and 18.3% and 0.6% and 19.7% respectively. With the Low Negative sample, the mean EpiScore was 18.5, and the reproducibility 3.39 (CV: 18.3%). With the High Negative sample, the mean EpiScore was 48.6, and the reproducibility 5.27 (CV: 10.8%). With the Low Positive sample, the mean EpiScore was 76.2, and the reproducibility 2.64 (CV: 3.5%). With the High Positive sample, the mean EpiScore was 96.7, and the reproducibility 0.17 (CV: 0.2%) ([table 9](#)).

Table 9 - Between lots precision and reproducibility - per sample type

Sample type	Mean	95% CI	Parameter	Value (SD)	95% CI	CV
Low negative	18.5	[15.8, 21.3]	Reproducibility	3.39	[2.47, 5.36]	18.3%
			Between lots precision	3.66	[1.94, 4.18]	19.7%
High negative	48.6	[44.9, 52.4]	Reproducibility	5.27	[4.13, 7.27]	10.8%
			Between lots precision	5.27	[3.50, 6.54]	10.8%
Low positive	76.2	[74.1, 78.2]	Reproducibility	2.64	[2.01, 3.85]	3.5%
			Between lots precision	2.98	[0.83, 3.83]	3.9%
High positive	96.7	[96.3, 97.1]	Reproducibility	0.17	[0.13, 0.25]	0.2%
			Between lots precision	0.55	[0.00, 0.29]	0.6%

Descriptive statistics were separately calculated for all sample combinations ([table 10](#)).

Table 10 - Simple Descriptive Statistics for EpiScore per Sample type – by Lot and Overall

Sample type	Lot combination	EpiScore					
		N	Mean	SD	Min	Median	Max
Low Negative	1	6	20.0	2.97	15	21	23
	2	6	15.2	3.54	11	16	21
	3	6	18.3	3.83	16	17	26
	4	6	19.3	3.20	16	19	23
	5	6	19.7	4.50	14	19	27
	All	30	18.5	3.83	11	18	27
High Negative	1	6	53.5	4.18	48	54	58
	2	6	47.7	4.80	41	48	54
	3	6	45.3	6.62	40	43	57
	4	6	48.8	6.37	41	50	56
	5	6	47.8	3.71	44	46	53
	All	30	48.6	5.60	40	49	58
Low Positive	1	6	76.2	5.64	65	79	80
	2	6	78.8	1.47	76	79	80
	3	6	75.2	1.94	74	75	79
	4	6	76.2	1.94	74	76	79
	5	6	75.0	1.67	73	75	77
	All	30	76.3	3.10	65	76	80
High Positive	1	6	97.0	0.00	97	97	97
	2	6	97.0	0.00	97	97	97
	3	6	96.5	0.84	95	97	97
	4	6	97.0	0.00	97	97	97
	5	6	96.3	0.52	96	96	97
	All	30	96.8	0.50	95	97	97

The study demonstrated that the Bladder EpiCheck results are reproducible across multiple reagent lots.

Bladder EpiCheck Assay Interference Testing

Interference was assessed for the two components of the Bladder EpiCheck kit, the Extraction Kit and the Test Kit using five common substances that potentially could be present in urine materials and interfere with the performance of the assay. Interfering substance, mechanism of interference and high/low sample types of the substances under test are specified in [table 11](#). Other materials which are not known as DNA-modifying substances and do not include human DNA that can create a signal in the Bladder EpiCheck assay were not tested since there is no reason to suspect that they will interfere with the Bladder EpiCheck assay in any way.

Table 11 – Interfering substances

Interfering substance	Mechanism of interference	Low concentration tested	High concentration tested
Hemoglobin	Potential inhibition of PCR reaction	0.5 mg/ml	1 mg/ml
Red blood cells	Potential inhibition of PCR reaction	5x10 ⁵ cells/mL	10 ⁶ cells/mL
Uric acid	Uric acid decreases the amount of DNA in the sample.	1.25 mg/ml	2.5 mg/ml
Ethanol	In high concentrations can interfere with DNA extraction	0.5% v/v	1% v/v
White blood cells	Includes human DNA that is a potential interfering substance due to a potential false signal in the PCR data.	5x10 ⁵ cells/mL	10 ⁶ cells/mL

Three voided urine pools (Healthy male, Healthy female, UCC male/female mix) were spiked with the substances specified in [table 9](#). Five replicate samples for each urine pool were evaluated for each substance and concentration (i.e., 30 samples per substance tested).

The sensitivity and specificity of the assay were 100.0% for all the substances and concentration tested. No failure to process was observed, except for uric acid when it was mixed with male urine pool. This might have happened due to the low amount of DNA in male urine compared to urine collected from females. Therefore, high levels of Uric acid present in urine collected from males may cause an Invalid result in the Bladder EpiCheck Assay due to interference. In case of Invalid result, the test should be repeated with a new urine sample. All other substances tested do not interfere with the Bladder EpiCheck assay.

4.2. Clinical Performance

Clinical Cutoff

The final cut-offs and the algorithm for the Bladder EpiCheck software were verified on a set of urine samples collected from control patients with a history of bladder cancer and bladder cancer positive patients confirmed by cystoscopy and pathology.

The total sample size of the dataset for the Bladder EpiCheck software algorithm development included 178 samples which were collected in two separate sets. The first set of samples served for the cut-off definition. The set included 109 samples, of which 40 were collected from control patients with a history of bladder cancer and 69 were collected from Urothelial Cell Carcinoma (UCC) positive patients confirmed by pathology. The derived Bladder EpiCheck algorithm sensitivity and specificity compared to pathology outcome demonstrated a sensitivity of 93% for UCC recurrence and specificity of 90%.

The second samples set served for the validation of the cut-off. The set included 67 samples, of which 51 were collected from control patients with a history of bladder cancer and 16 were collected from UCC positive patients confirmed by pathology. The derived Bladder EpiCheck algorithm sensitivity and specificity compared to pathology outcome demonstrated a sensitivity of 94% for UCC recurrence and specificity of 84%.

Clinical Performance - Bladder Cancer

Surveillance for recurrence of bladder cancer

Utility of Bladder EpiCheck for Detection of Recurrent Urothelial Carcinoma. A Multicenter, Prospective Blinded Study⁶.

Study Summary

A multicenter, prospective, single visit study was conducted at five (5) centers in Europe and Israel. The aim of the study was to determine the performance characteristics of the Bladder EpiCheck test for patients under surveillance for recurrence of bladder cancer. Data of 440 subjects was included in the analysis of the Bladder EpiCheck performance. The data was collected from subject coming for routine surveillance at the study centers from December 2015 until January 2017. The 'gold standard' used for all performance calculations was cystoscopy with pathology confirmation for positive or suspicious cystoscopies, and cytology. If patient had positive cystoscopy but pathology was absent the subject was considered positive based on the clinical decision of the treating urologist (e.g., initiation of MMC or BCG treatment). Subjects were omitted from analysis if no conclusive diagnosis was possible (e.g., equivocal cytology, cystoscopy or pathology).

The performance analysis included 353 subjects. The 87 excluded subjects include 30 urine samples with no Bladder EpiCheck results due to various reasons (e.g., insufficient amount of DNA in urine sample, urine sample damaged or got lost during transport), 50 subjects that were

omitted from analysis as no conclusive diagnosis was possible by the gold standard and 7 subjects with no Bladder EpiCheck and no conclusive diagnosis.

The Bladder EpiCheck assay was performed in two central labs located at Jena, Germany and Kfar-Saba, Israel by trained lab technicians according to the IFU.

The demographic data for the 440 subjects eligible to participate in the study are summarized in [table 12](#).

Table 12 – Subjects Demographics

Gender	N (%)
Male	341 (77.50%)
Female	99 (22.50%)
Race and Ethnicity	N (%)
Caucasian	440 (100%)
Not Hispanic or Latino	440 (100%)
Age	Years
Average	70.1
Range	31 - 92
Smoking History	N (%)
Never smoked	81 (18.41%)
Former smoker	252 (57.27%)
Currently smoking	107 (4.32%)

Bladder EpiCheck Performance vs. Gold Standard

353 subjects had complete data for performance analysis. Of the 353 subjects in the performance analysis 46 subjects were defined as positives. 40 were defined positive based on cystoscopy and pathology and another 6 based on cystoscopy and clinical decision of the treating urologist. Two (2) of the 46 positives don't have Bladder EpiCheck results. Hence, the total number of positives that were included in the performance analysis was 44.

309 subjects were defined as negatives based on cystoscopy and cytology results and 57 were omitted due to inconclusive diagnosis.

[Table 13](#) below presents the Bladder EpiCheck results versus the valid gold standard.

Table 13 – Bladder EpiCheck results versus the valid gold standard in NMIBC recurrence

Bladder EpiCheck result	Valid gold standard diagnosis (*)			
	Positive	Negative	Omitted(**)	Total
No EpiCheck result	2	28	7	37
Positive	30	37	16	83
Negative	14	272	34	320
Total	46	337	57	440

(*) Negative by cystoscopy and cytology / positive by cystoscopy and pathology or clinical decision

(**) No valid gold standard diagnosis

[Table 14](#) presents the stage and grade of the positive pathologies.

Table 14 – stage and grade of the positive pathologies in NMIBC recurrence

Stage	Grade			
	Total	Low Grade	High Grade	ND*
Total	46	21	19	6
Cis	3	0	3	
Ta	29	21	8	
T1	7		7	
T2	1		1	
ND*	6			

*Six positives are based on positive cystoscopy and clinical decision of the treating urologist. Two (2) of the 46 positives don't have Bladder EpiCheck results. The overall number of positives for analysis is hence 44.

[Table 15](#) presents the sensitivity and specificity of the Bladder EpiCheck test along with their respective two-sided, exact binomial, 95% CI's. Overall, the Bladder EpiCheck sensitivity observed was 68.18% (30/44), 95% CI: [52.42%;81.39%]. The Bladder EpiCheck sensitivity of all high-grade tumors was 91.67% (22/24), 95% CI: [73.00%;98.97%].

The specificity observed was 88.03% (272/309), 95% CI: [83.87%;91.43%].

Table 15 - Sensitivity and specificity of the Bladder EpiCheck in NMIBC recurrence

	% (n/N)	95% CI
Overall Sensitivity	68.18% (30/44)	[52.42%;81.39%]
Sensitivity of all positives except Ta Low grade	91.67% (22/24)	[73.00%;98.97%]
Specificity	88.03% (272/309)	[83.87%;91.43%]

[Table 16](#) presents the sensitivity of the Bladder EpiCheck by tumor stage and grade for all cases with pathology or clinical information available.

The Bladder EpiCheck kit showed greatest clinical sensitivity (100%) among the severe non muscle invasive tumors (T1 and CIS) compared to cystoscopy and pathology.

Table 16 - Sensitivity of the Bladder EpiCheck by tumor stage and grade in NMIBC recurrence

Stage	Clinical Sensitivity	95% CI
T1	100.0% (7/7)	[59.04%;100.0%]
CIS	100.0% (3/3)	[29.24%;100.0%]
Ta	51.85% (14/27)	[31.95%;71.33%]
Ta High Grade	85.71% (6/7)	[42.13%;99.64%]
Grade		
High Grade	91.67% (22/24)	[73.00%;98.97%]
Low Grade	40.00% (8/20)	[19.12%;63.95%]

[Table 17](#) presents the overall agreement, overall negative predictive value, negative predictive value of all subjects except of subjects with recurrence of Ta Low Grade, and positive predictive value. The overall negative predictive value was calculated based on the overall recurrence rate observed in the current study which was 12.2%. The negative predictive value of all subjects except of subjects with recurrence of Ta Low Grade was calculated based on the recurrence rate of high-grade tumors observed in the current study which was 7.2%.

Table 17 – Agreement, NPV and PPV in NMIBC recurrence

Parameter	% (n/N)	95% CI
Overall agreement	85.55% (302/353)	[81.45%;89.05%]
NPV	95.10% (272/286)	[91.92%;97.30%]
High grade tumors NPV	99.27% (272/274)	[97.39%;99.91%]
PPV	44.78% (30/67)	[32.60%;57.42%]

Aid in the detection of bladder cancer

Bladder EpiCheck Specificity in a population of urology patients without prior history or evidence of bladder cancer and healthy volunteers

A prospective multicenter study was performed in USA community urology centers to assess the specificity of Bladder EpiCheck test in a population of urology patients without prior history or evidence of bladder cancer and healthy volunteers. A total of 171 patients were enrolled to the study. Out of this cohort, 53 patients presented with either hematuria (microhematuria or macrohematuria) and/or Lower Urinary Tract Symptoms (LUTS). [Table 18](#) presents the demographics of the USA cohort

Table 18 – Demographics – USA cohort

Characteristic	
Age, median (range)	69 (27-94)
Sex, M /F %	110 (64.3%)/ 61 (35.7%)
Smoking history, Non-Smoker/ Former smoker/ Current Smoker	117 (68.4%)/ 40 (23.4%)/ 14 (8.2%)
Reason for visit	
LUTS, n (%)	41 (24.0%)
Urinary tract infection, n (%)	28 (16.4%)
Calculi, n (%)	18 (10.5%)
Hematuria, n (%)	14 (8.2%)
Other urological conditions, n (%)	80 (46.8%)
Healthy volunteer, n (%)	1 (0.6%)
Concurrent genitourinary cancer, n (%)	1 (0.6%) ^a
Concurrent non genitourinary cancer, n (%)	1 (0.6%) ^b

(a) Vaginal cancer; (b) Breast cancer

A total of 150 patients had a valid Bladder EpiCheck result, 3 of which were positive, leading to specificity of 98.0% (94.3%-99.6%). In the subset of patients with hematuria or LUTS, specificity

was similar 97.8% (88.5%-99.9%). Presence of hematuria, UTI or calculi did not influence the specificity ($p>0.45$ by Fisher Exact test). [Table 19](#) presents the specificity by subgroup of the USA cohort

Table 19- Bladder EpiCheck specificity in bladder cancer detection by subgroup – USA cohort

	N	Bladder EpiCheck negative	Specificity (CI95%)
All negative patients	150	147	98.0% (94.3%-99.6%)
LUTS and/or hematuria	46	45	97.8% (88.5%-99.9%)
UTI	27	26	96.3% (81.0%-99.9%)
Calculi	15	15	100.0% (78.2%-100.0%)

Another prospective single center study was performed in a Spanish urology outpatient clinic to assess the specificity of Bladder EpiCheck test in a population of urology patients without prior history or evidence of bladder cancer presenting with hematuria. A total of 46 patients were enrolled to the study, out of which 31 (67.4%) were males. [Table 20](#) presents the clinical details of the Spanish cohort

Table 20 – Clinical details – Spanish cohort

Characteristic	
Diagnosis	
Calculi, n (%) ^a	30 (65.2%)
BPH, n (%) ^a	12 (26.1%)
Urinary tract infection, n (%)	4 (8.7%)
Other urological conditions, n (%)	1 (2.2%)
Level of hematuria in the collected urine sample by Roche Combur-Test®	
1	4 (8.7%)
2	1 (2.2%)
3	3 (6.5%)
4	22 (47.8%)
Negative	16 (34.8%)

(a) one patient had both calculi and BPH

A total of 43 patients had a valid Bladder EpiCheck result, and all were negative, leading to specificity of 100.0% (91.8-100.0%). [Table 21](#) presents the specificity of the Spanish cohort

Table 21- Bladder EpiCheck specificity in bladder cancer detection - Spanish cohort

	N	Bladder EpiCheck negative	Specificity (CI95%)
All negative patients	43	43	100.0% (91.8-100.0%)
Calculi^a	29	29	100.0% (88.1-100.0%)
BPH^a	11	11	100.0% (71.5-100.0%)
UTI	3	3	100.0% (29.2-100.0%)

(a) one patient had both calculi and BPH

Bladder EpiCheck Sensitivity in Patients with Primary Bladder Cancer

In addition to the specificity studies, a prospective single center study was performed in Spain to assess the sensitivity of Bladder EpiCheck test in patients with primary bladder cancer. A total of 64 patients with newly diagnosed bladder cancer were enrolled to the study, diagnosis was confirmed by pathology for all. A urine sample was collected prior to TURBT. Median age was 74 (range 43-96) and 56 (87.5%) were males. A total of 62 patients had a valid Bladder EpiCheck result, 41 of which were positive, leading to overall sensitivity of 66.1% (53.0%-77.7%). Overall, 40 patients were diagnosed with high-grade tumors. The sensitivity for the High-grade tumors was 87.5% (73.2%-95.8%), which was similar across all stages ($p=0.5135$ by Fisher Exact test). Low-grade sensitivity was 27.3% (10.7%-50.2%). [Table 22](#) presents the overall sensitivity and sensitivity by disease grade and stage.

Table 22- Bladder EpiCheck sensitivity in bladder cancer detection, overall and by disease grade and stage

	N	Bladder EpiCheck Positive	Sensitivity (CI95%)
All Positive patients	62	41	66.1% (53.0%-77.7%)
High-grade^a	40	35	87.5% (73.2%-95.8%)
Ta	18	17	94.4% (72.7%-99.9%)
T1	14	13	92.9% (66.1%-99.8%)
T2	5	4	80.0% (28.4%-99.5%)
Concomitant CIS^b	15	14	93.3% (68.1%-99.8%)
Low-grade or PUNLMP	22	6	27.3% (10.7%-50.2%)

(a) Five patients did not have stage information: 3 low-grade and 2 high-grade. (b) Ten patients did not have concomitant CIS information

Bladder EpiCheck NPV and PPV in bladder cancer detection in various scenarios of disease prevalence, according to overall sensitivity of 66.1%, HG sensitivity of 87.5% and specificity of 98%. [Table 23](#) presents the calculated NPV and PPV for bladder cancer detection.

Table 23- Bladder EpiCheck calculated NPV and PPV for bladder cancer detection

Prevalence of disease	1% prevalence	3% prevalence	10% prevalence
Overall NPV	99.7%	98.9%	96.3%
HG NPV	99.9%	99.6%	98.6%
PPV	25.0%	50.5%	78.6%

Clinical Performance – Upper Tract Urothelial Carcinoma

Aid in the Detection and Monitoring of Recurrence of Upper Tract Urothelial Carcinoma

Two independent publications reported the performance of Bladder EpiCheck in the detection and monitoring of tumor recurrence of upper tract urothelial carcinoma (UTUC). Overall, 229 patients were included in the reported studies as follows:

Gallioli et al.⁷ prospectively enrolled 95 patients, of which 83 underwent at least 1 urine collection and had a valid Bladder EpiCheck result. Median age was 72 (64-78) and 63 (75.9%) were males. 64 (77.1%) of the patients were referred to the clinic for assessment for the presence of UTUC due to suspicious symptoms or findings, and 19 (22.9%) were followed-up for a known UTUC. Many patients had history of urothelial carcinoma, 46 (55.4%), 39 (47.0%) and 21 (25.3%) had previous bladder cancer, previous homolateral or contralateral UTUC, respectively. Urine for Bladder EpiCheck and cytology was collected from the bladder in patients with intact bladder (n=72) and from one or both of the ureters (n=82). The results of the tests were compared with ureteral biopsy as reference standard. Patients were excluded from the analysis if they had a metachronous bladder tumor and if Bladder EpiCheck did not yield a result.

Pierconti et al.⁸ performed a retrospective analysis of 134 patients, 82 patients with clinically localized high-grade UTUC who had undergone a radical nephroureterectomy and 52 patients presented with hematuria which was determined as negative for bladder cancer and UTUC by cystoscopy, cytology and follow-up. The confirmed UTUC patients were mostly male 55 (67%) with a median age of 70.5 (45-93), no demographics data is reported for the control group. Urine for Bladder EpiCheck and cytology was collected from the ureter in both groups by selective ureteral catheterization prior to any surgical procedure, if performed. In the control patients, 20 (38.5%) were clinically diagnosed with stones and the rest 32 (61.5%) with inflammatory condition of the upper tract. Patients were excluded from the analysis if Bladder EpiCheck did not yield a result. The results of the two studies are summarized in [Tables 24](#) and [25](#).

Table 24- Bladder EpiCheck performance in UTUC - summary of both cohorts

	Gallioli et al. ⁷		Pierconti et al. ⁸	
Urine source	Bladder EpiCheck	Cytology	Bladder EpiCheck	Cytology
Ureter, n	72		134	
Sensitivity	84.8%	63.0%	NA	NA
HG sensitivity	95.8%	80.0%	97.4% (91.0%-99.7%)	59.0% (47.3%-70.0%)
Specificity	80.6%	91.7%	100.0% (93.0%-100.0%)	96.0% (86.3%-99.5%)
Bladder, n	82			
Sensitivity	58.5%	37.5%		
HG sensitivity	73.7%	57.9%		
Specificity	80.6%	93.7%		

(a) CI95% was not reported

Bladder EpiCheck HG NPV by source of urine in various scenarios of disease prevalence, (based on Gallioli et al.)

Table 25- Bladder EpiCheck calculated NPV in UTUC

HG NPV	1% prevalence	3% prevalence	10% prevalence
Urine from the ureter	99.95%	99.84%	99.42%
Urine from the bladder	99.67%	99.00%	96.5%

5. Reagents

5.1. Bladder EpiCheck DNA Extraction Kit (P/N NX899090-01C)

This kit contains reagents and consumables for DNA extraction from cell pellet of urine samples. Reagents are sufficient for extraction of 50 samples.

Table 26 - Reagents supplied in the Bladder EpiCheck DNA Extraction Kit

Reagent	Volume / Amount
Mini Spin Columns with collection tube (2ml)	50
Collection Tubes (2ml)	4X50
Safe-Lock Tubes (1.5ml)	75
Buffer NL*	2X12ml
Buffer NW1* (concentrate)	19ml
Buffer NW2 [†] (concentrate)	13ml
Buffer NE	15ml
Protease (lyophilized)	1 vial [‡]
Protease Solvent [†]	1.2ml

* Contains chaotropic salt. Not compatible with disinfecting agents containing bleach; see [page 29](#) for safety information.

† Contains sodium azide as a preservative.

‡ Resuspension volume 1.2 ml.

5.2. Bladder EpiCheck Test Kit (P/N NX899090-02C)

This kit contains reagents for the digestion and the real-time PCR amplification assay. Reagents are sufficient for total of 60 reactions. Each run requires 2 control reactions: UC and NTC. For efficient use of reagents, consider running full plates (10 samples + 2 controls per plate), meaning that the reagents are sufficient for a total of 50 patient samples + 10 controls.

Table 27 - Reagents supplied in the Bladder EpiCheck Test Kit

Color	Reagent	Ingredients	Volume
Yellow	PCR mix – A	IR primers and probe, BE-1 primers and probe, BE-2 primers and probe, PCR buffer, dNTPs	1100µl
Yellow	PCR mix - B	IR primers and probe, BE-3 primers and probe, BE-4 primers and probe, PCR buffer, dNTPs	1100µl
Yellow	PCR mix - C	IR primers and probe, BE-5 primers and probe, BE-6 primers and probe, PCR buffer, dNTPs	1100µl
Yellow	PCR mix - D	IR primers and probe, BE-7 primers and probe, BE-8 primers and probe, PCR buffer, dNTPs	1100µl
Yellow	PCR mix - E	IR primers and probe, BE-9 primers and probe, BE- 10 primers and probe, PCR buffer, dNTPs	1100µl
Yellow	PCR mix - F	IR primers and probe, DC primers and probe, BE-11 primers and probe, DCR primers and probe, PCR buffer, dNTPs	1100µl
Yellow	PCR mix - G	IR primers and probe, BE-12 primers and probe, BE-13 primers and probe, PCR buffer, dNTPs	1100µl
Yellow	PCR mix - H	IR primers and probe, BE-14 primers and probe, BE-15 primers and probe, PCR buffer, dNTPs	1100µl
Orange	Taq DNA Polymerase [5U/µl]	Taq DNA Polymerase, Tris-HCl, KCl, EDTA, DTT, 50% Glycerol (v/v), 0.5% Tween®,	6X50µl
White	10X Digestion Buffer	Potassium acetate, Tris-acetate, Magnesium acetate, BSA	2X1250µl
Light Purple	1X Dilution Buffer	Potassium acetate, Tris-acetate, Magnesium acetate, BSA	1500µl
White	Digestion Enzyme [10U/µl]	KCl, Tris-HCl, DTT, EDTA, 50% Glycerol (v/v), BSA	2X220µl
Purple	Digestion Control Template [100fg/µl]	Exogenous sequences DC and DCR dissolved in NE buffer	150µl
White	NTC (No Template Control)	Ultra pure water, DNA free	1600µl
Blue	UC (Undigested Control) [2fg/µl]	17 exogenous sequences dissolved in NE buffer	1600µl

5.3. Platform

The Bladder EpiCheck kit is specifically designed for use with the following platforms together with the Bladder EpiCheck software:

1. Applied Biosystems (ABI) 7500 Fast Dx real time PCR instrument with the Sequence Detection Software version 1.4.
2. QIAGEN Rotor Gene Q and Rotor Gene Q MDx real time PCR instrument platforms:
 - a. 5plex
 - b. 5plex HRM
 - c. 6plex

With the Rotor Gene Q Series Software version 2.3.1 and the 72-well rotor.

For the latest version of the Bladder EpiCheck software visit our website

www.nucleix.com/customer-area/

Please refer to the instrument user manual for information concerning the instrument. The Applied Biosystems (ABI) 7500 Fast Dx real time PCR instrument must be maintained according to the requirements in the instrument user manual.

Please follow instructions for installing and analyzing data using Bladder EpiCheck software ([page 47](#)).

Note: This document is the IFU for the ABI 7500 Fast Dx platform. If you wish to use the QIAGEN Rotor Gene Q platform, please download its corresponding IFU from our website at

www.nucleix.com/customer-area/

6. Reagent Storage and Handling

6.1. Bladder EpiCheck DNA Extraction Kit (P/N NX899090-01C)

1. Mini spin columns should be stored at 2–8°C upon arrival and can be used until the expiration date on the kit box.
2. All buffers can be stored at room temperature (15–25°C) until the expiration date on the kit box.
3. Lyophilized Protease can be stored at room temperature (15–25°C) until the kit expiration date without affecting performance. Reconstituted protease is stable for up to 1 year when stored at 2–8°C, but only until the kit expiration date.
4. Reconstituted Wash Buffer 1 and reconstituted Wash Buffer 2 (NW2) are stable for up to 1 year when stored at room temperature (15–25°C), but only until the kit expiration date.

6.2. Bladder EpiCheck Test Kit (P/N NX899090-02C)

1. All reagents of Bladder EpiCheck Test Kit should be stored in -20°C.
2. Thaw only the required number of Taq DNA Polymerase tubes.
3. When using the Digestion enzyme always insert tube into ice or place in a Mini Cooler.

6.3. Important issues before getting started

1. After receiving Bladder EpiCheck kit, check that kit components are not damaged. If the blister packs of the mini spin columns or the buffer bottles are damaged, contact your local distributor. In case of liquid spillage, refer to the Safety Information section ([page 29](#)). Do not use damaged kit components.
2. Make sure you install the Bladder EpiCheck software. See section 11.1 ([page 47](#)) for installation instructions.
3. To minimize cross-contamination, always change tips between each step of liquid transfer. Always use tips containing aerosol barrier. Use disposable gloves and change them whenever they might have been contaminated.

7. Warnings and Precautions

- The Bladder EpiCheck test is designed to be used with ABI 7500 Fast Dx and QIAGEN Rotor gene Q instruments only.
- When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective glasses. For more information, please consult the appropriate safety data sheets (SDSs). These are available online at www.nucleix.com/customer-area/

7.1. Safety Information

Cell pellet preparation waste

When handling urine samples always wear a suitable lab coat, disposable gloves, and protective glasses. Use a bio-hazard container for cell pellet preparation waste.

Dispose cell pellet preparation waste as well as urine samples containers in accordance with national/local regulations relating to Bio-hazard waste disposal.

If the cell pellet preparation waste is spilt: (i) avoid contact with skin and eyes and wear appropriate protective clothing, gloves, facemask, and glasses, (ii) absorb the material, (iii) clean the surface with 10% (v/v) sodium hypochlorite and leave the surface wet for one hour, (iv) wash thoroughly with water, (v) place the waste in suitable container and dispose of in accordance with national/local regulations relating to special waste disposal.

Bladder EpiCheck DNA Extraction Kit (P/N NX899090-01C)

When performing DNA extraction, always wear a suitable lab coat, disposable gloves, protective glasses and mask. The Lysis Buffer (NL) and Wash Buffer 1 (NW1) contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite. If the buffer bottles are damaged or leaking, wear gloves and protective glasses when discarding the bottles in order to avoid personal injury or injury to others.

Liquid waste must be considered infectious and be handled and discarded according to national/local regulations relating to chemical waste disposal.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

The following hazard and precautions statements apply to components of the Bladder EpiCheck DNA Extraction Kit:

Lysis Buffer (NL)



Contains guanidine hydrochloride, maleic acid: harmful, irritant. Hazard and precautionary statements:

(i) **Hazard:** H315 Causes skin irritation; H317 May cause an allergic skin reaction; H319 Causes serious eye irritation; (ii) **Prevention:** P280 Wear protective gloves/ protective clothing/ eye protection/ face protection

Wash Buffer 1 (NW1)



Contains guanidine hydrochloride: harmful, irritant. Hazard and precautionary statements:

(i) **Hazard:** H302 + H332 Harmful if swallowed or if inhaled; H315 Causes skin irritation; H319 Causes serious eye irritation. (ii) **Prevention:** P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.

Protease (lyophilized)



Contains subtilisin: sensitizer, irritant. Hazard and precautionary statements:

(i) **Hazard:** H318 Causes serious eye damage; H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled. (ii) **Prevention:** P261 Avoid breathing dust/ fume/ gas/ mist/ vapors/ spray; P280 Wear protective gloves/ protective clothing/ eye protection/ face protection; P284 Wear respiratory protection; (iii) **Response:** P304 + P341 if inhaled: if breathing is difficult, remove victim to fresh air and keep at rest in a position comfortable for breathing; P305 + P351 + P338 if in eyes: rinse cautiously with water for several minutes, Remove contact lenses, if present and easy to do. Continue rinsing; P308 + P311 if exposed or concerned: call a poison center or doctor/ physician; P342 + P311 If experiencing respiratory symptoms: call a poison center or doctor/ physician.

Bladder EpiCheck Test Kit (P/N NX899090-02C)

The Bladder EpiCheck Test Kit contains no substances which at their given concentration, are considered to be hazardous to health. We recommend handling all chemicals with caution, always wear a suitable lab coat, disposable gloves, and protective glasses.

PCR plates containing DNA and Control samples – dispose PCR plates with DNA and control samples as bio-hazard in accordance with national/local regulations relating to Bio-hazard waste disposal.

Dispose of “UC” and “Digestion control template” tubes as bio-hazard in accordance with national/local regulations relating to Bio-hazard waste disposal.

8. Materials Required but not Provided

8.1. Reagents

- Phosphate Buffered Saline (PBS) 0.01M pH 7.4 (e.g. Sigma cat# P3813 or Thermo Fisher cat# A1286301)
- Ethanol (96-100%). Do not use denatured alcohol, which contains other substances
- Double distilled water (1L bottles) in case PBS by Sigma is being used.

8.2. Consumables

- Pipette tips with aerosol barrier
- 50ml conical tubes
- MicroAmp[®] Fast Optical 96-Well Reaction Plate with Barcode, 0.1 ml (Applied Biosystems, Life Technologies Co. cat# 4346906) or MicroAmp[®] Fast Optical 96-Well Reaction Plate without Barcode, 0.1 ml (Applied Biosystems, Life Technologies Co. cat# 4346907)

Note: Only the above specified 96-well plates should be used to perform the test.

- MicroAmp[®] 96- & 384-Well Optical Adhesive Film (Applied Biosystems, Life Technologies Co. cat# 4311971)

Note: Only the above specified optical adhesive film should be used to perform the test.

8.3. Equipment

- ABI 7500 Fast Dx real time PCR instrument with Sequence Detection Software version 1.4.
- Microcentrifuge with rotor for 1.5ml tubes, able to reach at least 12,000g (not a benchtop mini centrifuge)
- Centrifuge with a 96 well plate rotor
- Centrifuge with a swing bucket rotor for 50ml conical tubes, able to switch the break off
- Dry bath / Heating block suitable for 37°C and 65°C (suitable for 1.5ml tubes)
- Pipettes (adjustable volume) for sample preparation
- Container with ice / Mini Cooler for 1.5ml centrifuge tubes (-20°C)
- Permanent marker
- Vortexer

9. Specimen Collection, Transport and Storage

The Bladder EpiCheck kit is designed to be used on urine samples.

Please take into consideration that the maximum time between specimen collection and the cell pellet preparation at the laboratory is 5 days, during which the specimen is refrigerated and may be in room temperature (15-25°C) for a maximum of 24 hours (for transportation).

9.1. Specimen collection procedure

1. Collect a voided urine sample from the patient, minimal volume required to process is 10 ml. Samples with a higher volume have a higher success rate.
2. Specimens are to be stored at 2-8°C until transportation to the laboratory. **Do not add preservatives to the sample.**
3. Transport specimens to the laboratory. Time on the road should not exceed 24 hours if the specimen is not shipped refrigerated.
Note: in case urine samples are transferred from the collection container to a secondary container (for example 50ml tube) please perform the following step:
Mix thoroughly the specimen container such that the sample is homogenous and then transfer the urine to the new container.
4. Upon arrival to the laboratory store specimens at 2-8°C. Processing of urine sample should be performed no longer than 5 days from the time of collection.

9.2. Special precautions

1. Urine samples containers should be marked and handled as Bio-hazard according to local handling procedures.
2. Always use protective gloves and a lab coat when handling samples.
3. Make sure the containers are tightly closed and intact before shipment.
4. Make sure samples are labeled properly.

10. Procedure

10.1. Cell pellet preparation

Things to do before starting:

1. If PBS by Sigma is being used, prepare PBS 0.01M pH 7.4 solution [1 liter is sufficient for approximately 15 samples].

Cell pellet preparation procedure:

1. Mix thoroughly the specimen container such that the sample is homogenous.
2. Transfer 10-50ml urine to a 50ml centrifuge tube. For centrifugation adjust volume with PBS 0.01M pH 7.4.
Note: Maximal urine to be processed is 50ml.
3. Centrifuge at 1000g for 10 min at room temperature (15–25°C) and turn off the centrifuge brake.
4. Carefully discard urine into a suitable Bio-hazard waste container and make sure not to drop the pellet.
5. Resuspend the pellet in the residual urine left in the tube and add 40ml PBS 0.01M pH 7.4.
6. Mix the pellet with the PBS by inverting the tube a few times to ensure efficient wash of the cells.
7. Centrifuge at 1000g for 10 min at room temperature (15–25°C) and turn off the centrifuge brake.
8. Carefully discard PBS into a suitable Bio-hazard waste container and make sure not to drop the pellet.
9. Resuspend the cell pellet in the remaining PBS in the tube and transfer the entire pellet volume to a 1.5ml microcentrifuge tube.
10. Use the marked lines on the tube to visually inspect the resuspended cell pellet volume. The required volume for the DNA extraction step is ~400µl. If the volume is in the range of 400-500µl, use the entire volume in the DNA extraction step.
11. If needed, use the following instructions to adjust the volume to ~400µl:
 - i. If the volume is <400µl, add PBS 0.01M pH 7.4 until reaching approximately 400µl.
 - ii. In case the volume is >500µl, centrifuge the tube at 1000g for 1 min and carefully discard the supernatant until reaching approximately 400µl.

Note: Pellet is not always visible, proceed with DNA extraction protocol even if you cannot see the pellet.

General note: if you don't proceed immediately to step 10.2 (DNA extraction), pellet samples can be kept for up to 14 days at –20°C.

10.2. DNA extraction

Use Bladder EpiCheck DNA Extraction Kit (NX899090-01C)

Things to do before starting:

1. Prepare Buffer NW1, Buffer NW2, and Protease according to the following instructions (only done once in the first use of Bladder EpiCheck DNA Extraction Kit):
2. **Protease stock solution:**
Add 1.2 ml Protease Solvent to the vial of lyophilized Protease and mix carefully by inverting the vial several times to avoid foaming. Make sure that the Protease is completely dissolved. Store at 2-8°C.
3. **Buffer NL:**
Mix Buffer NL thoroughly by shaking before use. If a precipitate has formed in Buffer NL, dissolve it by incubating at 56°C. Store at 15-25°C
Note: Do not add Protease directly to Buffer NL.
4. **Buffer NW1:**
Using a sterile disposable pipette, add 25 ml ethanol (96–100%) to the bottle containing 19 ml Wash Buffer 1 (NW1) concentrate. Store the reconstituted Wash Buffer 1 (NW1) at room temperature (15–25°C).
Reconstitute Wash Buffer 1 (NW1) by inverting the bottle several times before starting the procedure.
5. **Buffer NW2:**
Using a sterile disposable pipette, add 30 ml ethanol (96–100%) to the bottle containing 13 ml Wash Buffer 2 (NW2) concentrate. Store the reconstituted Wash Buffer 2 (NW2) at room temperature (15–25°C).
Reconstitute Wash Buffer 2 (NW2) by inverting the bottle several times before starting the procedure.

DNA Extraction Procedure:

Please notice:

1. Change tips between each sample in each step of the protocol.
2. Use the provided safe-lock microcentrifuge tubes to elute the DNA (step 15).

1. **If the cell pellet sample is frozen, thaw it at room temperature (15-25°C) and briefly spin before use.**
2. **Take out the appropriate number of Mini centrifuge columns from 2-8 °C, according to the number of the pellet samples.**
3. **Add 20µl Protease stock solution to each sample.**
4. **Add 400µl Buffer NL to each sample and mix immediately by vortexing for 15 s.**
To ensure efficient lysis, it is essential that the sample and Buffer NL are mixed immediately and thoroughly.
Note: Do not add Protease directly to Buffer NL.

5. Incubate at room temperature (15–25°C) for 10 min. Briefly centrifuge to remove drops from inside the lid.
6. Add 400µl ethanol (96–100%) to the sample and mix again by vortexing. Briefly centrifuge to remove drops from inside the lid.
7. Carefully apply 700µl of the mixture from step 6 to a Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g for 1 min. [Do not discard the rest of the sample].
Transfer supernatant only. Do not load viscous pellet or any particulates to the mini spin column to avoid clogging the membrane.
Close each spin column to avoid aerosol formation during centrifugation.
8. Place the Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate¹.
9. Repeat step 7 by applying the remaining mixture from step 6 (~500µl) to the Mini spin column. Close the cap and centrifuge at 6000 x g for 1 min.
Transfer supernatant only. Do not load viscous pellet or any particulates to the mini spin column to avoid clogging the membrane.
10. Place the Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
11. Carefully open the Mini spin column and add 500µl Buffer NW1 without wetting the rim. Close the cap and centrifuge at 6000 x g for 1 min.
12. Place the Mini spin column in a clean 2ml collection tube and discard the collection tube containing the filtrate¹.
13. Carefully open the Mini spin column and add 500µl Buffer NW2 without wetting the rim. Close the cap and centrifuge at full speed (17,000 x g) for 3 min.
14. Place the Mini spin column in a new 2ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed (17,000 x g) for 1 min.
This step helps to eliminate the chance of possible Buffer NW2 carryover.
Note: only a very small drop should be left at the bottom of the collection tube in the end of this step. In case of a higher filtrate volume, place the Mini spin column in a clean 2 ml collection tube and centrifuge again at full speed (17,000 x g) for 1 min.
15. Place the Mini spin column in a clean 1.5ml safe-lock microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the Mini spin column and add 100µl Buffer NE.
16. Incubate at room temperature (15–25°C) for 1min, and then centrifuge at 10,000 x g for 2 min.
17. The filtrate contains the eluted DNA, discard the Mini spin column.

Note: if you don't proceed to step 10.3 (DNA digestion), DNA samples can be kept for up to 30 days at -20°C.

¹ Flow-through contains Buffer NL and is therefore not compatible with bleach. See [page 29](#) for safety information.

10.3. DNA digestion

Use Bladder EpiCheck Test Kit (NX899090-02C)

This step is performed on the entire 100µl eluate of the DNA samples inside the original elution microcentrifuge tube.

Thing to do before starting:

- In each run a maximum of 10 patient samples and 2 controls can be ran, meaning that for every 10 patient samples prepare one UC and one NTC digested samples. For example, if you have 14 patient samples prepare 2 UC samples and 2 NTC samples for digestion.
- Prepare a container with ice.
- Prepare dry bath for 37°C.

Note : Change tips between each sample in each step of the protocol.

1. Completely thaw the following tubes at room temperature (15-25°C):
 - a. DNA samples (if they were frozen)
 - b. Digestion buffer
 - c. Digestion control template
 - d. UC and NTC vials
2. Once thawed, mix each tube thoroughly by inverting and flicking the tube a few times, **do not vortex**. Briefly spin down to remove drops from inside the lid.
3. Transfer 100µl of UC sample and 100µl of NTC sample to two separate clean safe-lock microcentrifuge tubes (provided in the DNA Extraction Kit NX-899090-01).
4. Take one vial of digestion enzyme and place it on ice. Before use mix the tube thoroughly by inverting and flicking it a few times, **do not vortex**. Briefly spin down to remove drops from inside the lid.
5. Prepare digestion mix according to [table 28](#) below (in order):

Table 28 - Preparation of digestion reaction mix

Vial	Volume (µl)
10X Digestion buffer	12µl x (n+1) *
Digestion control template	2 µl x (n+1) *
Digestion enzyme	6µl x (n+1) *
Total volume	20µl x (n+1)*

* **n** is the number of reactions including NTC and UC samples. For example, if you have 5 patient samples then n = 8.

6. Mix the prepared digestion reaction mix (do not vortex), and briefly spin down to remove drops from inside the lid.
7. Add 20 µl of the digestion reaction mix to each DNA sample and to NTC and UC samples tubes, change tips between samples. Close cap and mix by inverting the tube a few times (do not vortex), and briefly spin down to remove drops from inside the lid.
8. Place tubes in a dry bath according to the following thermal conditions ([table 29](#)). Make sure that the tube lids are completely locked to avoid evaporation.

Table 29 - Digestion reaction thermal conditions*

Temperature (°C)	Duration (min)
37	120
65	20

* Allow the sample tubes to remain in the dry bath while heating to 65°C.

9. Briefly spin down the tubes to remove drops from inside the lid.

Note: if you don't proceed immediately to step 10.4 (real-time PCR), freeze digested DNA tubes at -20°C. Samples can be kept for up to 30 days.

10.4. Real-time PCR

Use Bladder EpiCheck Test Kit (NX899090-02C)

Note: Use only the specified 96-well plates and optical adhesive films (see page 32, Materials Required But not provided, point 8.2 Consumables)

1. Completely thaw the following tubes at room temperature (15-25°C):
 - a. Digested DNA samples (if they were frozen)
 - b. PCR mixes A-H (8 vials)
 - c. Taq DNA polymerase (according to the required volume calculated in [table 30](#) below)
2. Once thawed, mix each tube thoroughly by inverting the tube a few times, then briefly spin down to remove drops from inside the lid. **Do not vortex.**
3. Prepare 8 clean 1.5ml microcentrifuge tubes and prepare assay mixes A-H according to [table 30](#), using the matching PCR mixes A-H (e.g. for assay mix A use PCR mix A). Change tips between each mix.

Table 30 - Preparation of assay mixes A-H

Assay mix	Volume of PCR mix	Volume of Taq DNA Polymerase	Total Volume
A	14.5µl x (n+1)*	0.5µl x (n+1)*	15µl x (n+1)*
B	14.5µl x (n+1)*	0.5µl x (n+1)*	15µl x (n+1)*
C	14.5µl x (n+1)*	0.5µl x (n+1)*	15µl x (n+1)*
D	14.5µl x (n+1)*	0.5µl x (n+1)*	15µl x (n+1)*
E	14.5µl x (n+1)*	0.5µl x (n+1)*	15µl x (n+1)*
F	14.5µl x (n+1)*	0.5µl x (n+1)*	15µl x (n+1)*
G	14.5µl x (n+1)*	0.5µl x (n+1)*	15µl x (n+1)*
H	14.5µl x (n+1)*	0.5µl x (n+1)*	15µl x (n+1)*

* **n** is the number of reactions including 2 controls (UC and NTC). For example, if you have 5 patient samples then n = 7.

4. Mix each tube thoroughly by inverting the tube a few times, briefly spin down to remove drops from inside the lid, **do not vortex.**
5. Take a MicroAmp® 96 well PCR plate, and pipette 15µl of assay mix A into row A, 15µl of assay mix B into row B etc., see [Figure 1](#). Change tips between mixes to avoid cross reactions.

Figure 1 - Pipetting of assay mixes A-H to a 96-well PCR plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	A	A	A	A	A	A	A	A	A	A
B	B	B	B	B	B	B	B	B	B	B	B	B
C	C	C	C	C	C	C	C	C	C	C	C	C
D	D	D	D	D	D	D	D	D	D	D	D	D
E	E	E	E	E	E	E	E	E	E	E	E	E
F	F	F	F	F	F	F	F	F	F	F	F	F
G	G	G	G	G	G	G	G	G	G	G	G	G
H	H	H	H	H	H	H	H	H	H	H	H	H

- Pipette 10µl of each digested DNA sample and controls (from step 10.3) according to [Figure 2](#) below. Change tips between each well.

Note: Keep the residual volume of the digested DNA samples until a successful analysis using the Bladder EpiCheck® software is obtained. It might be further used in case of a failure (See page 72, Protocol for Repeating PCR). Samples should be stored at -20°C.

Figure 2 - Sample pipetting scheme

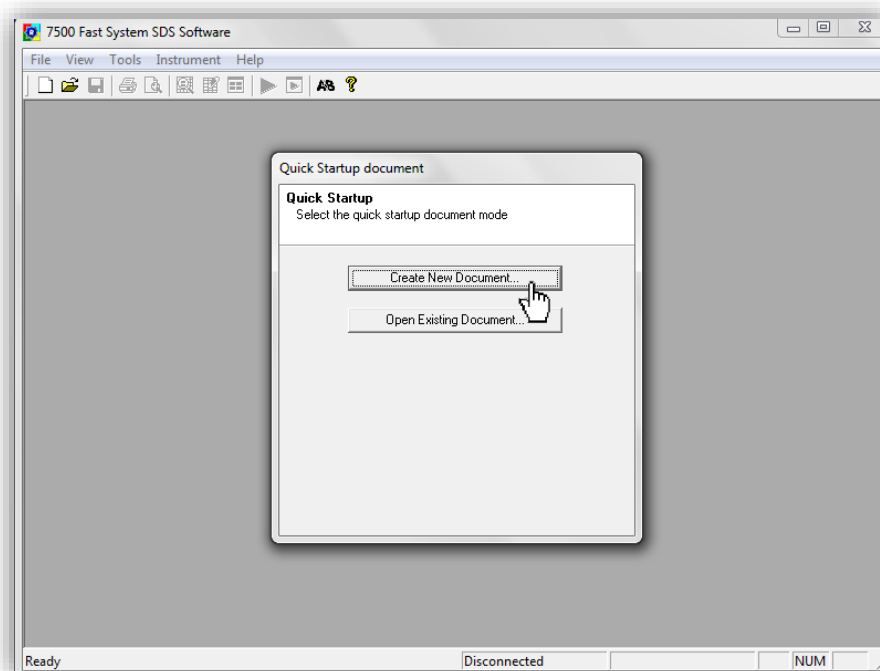
	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	UC	NTC
B												
C												
D												
E												
F												
G												
H												

Note: Figure 2 illustrates a full plate including 10 samples, however a smaller number of samples can also be used

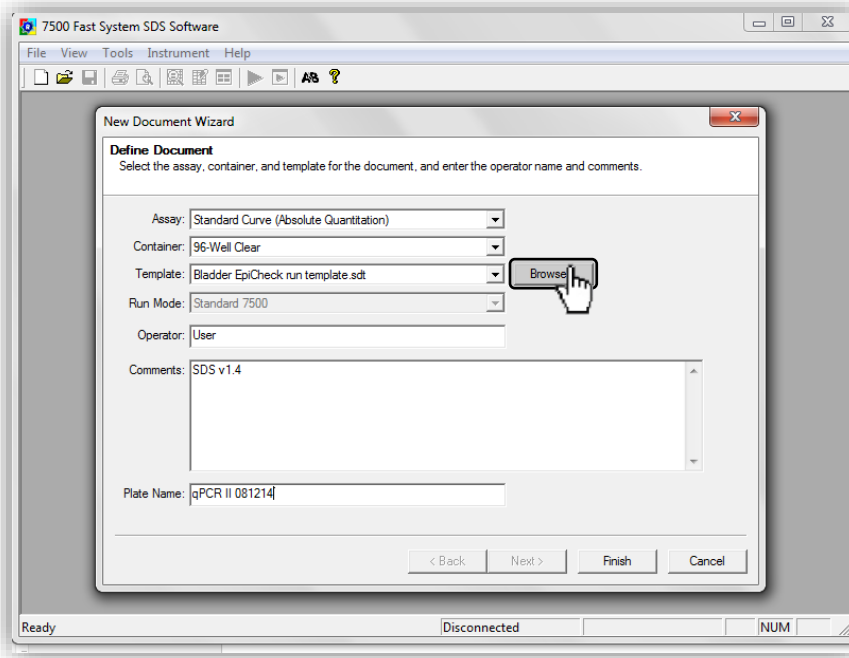
- Cover the plate with MicroAmp® 96- & 384-Well Optical Adhesive Film. Make sure it is tightly sealed to avoid evaporation.
- Briefly centrifuge the plate.

PCR run set-up in ABI 7500 fast DX instrument:

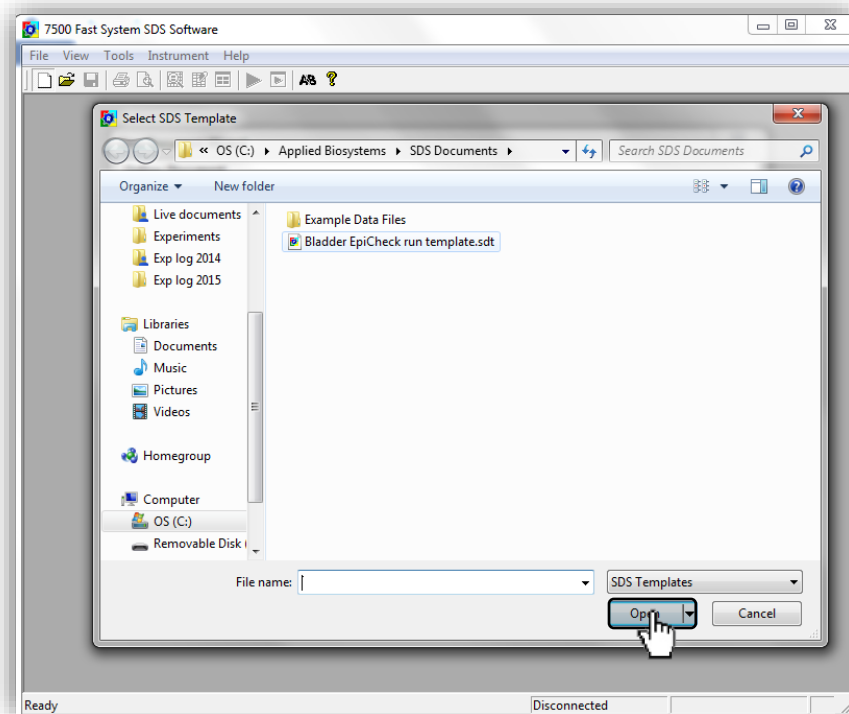
1. Turn on the ABI 7500 Fast Dx instrument and computer.
2. Turn on 7500 fast system software (software Icon: )
3. Insert the PCR plate into the instrument.
4. Choose “*Create New Document*” from the quick startup wizard:



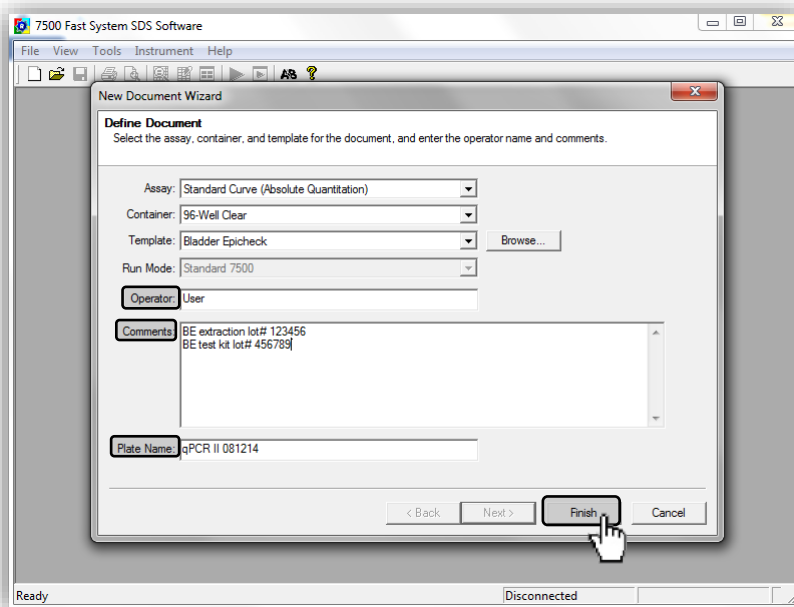
- In the “New Document Wizard” click on “Browse”:



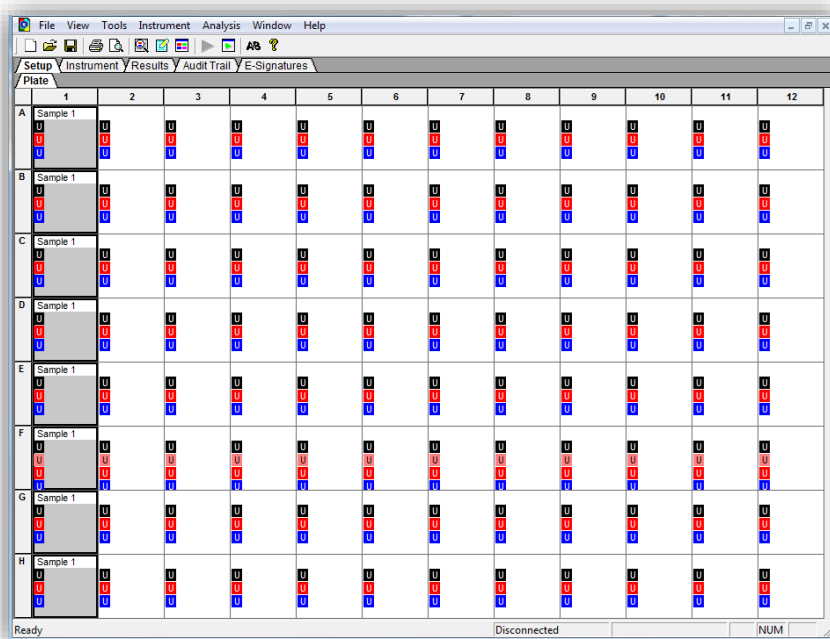
- Select the “Bladder EpiCheck run template” then click “Open”.



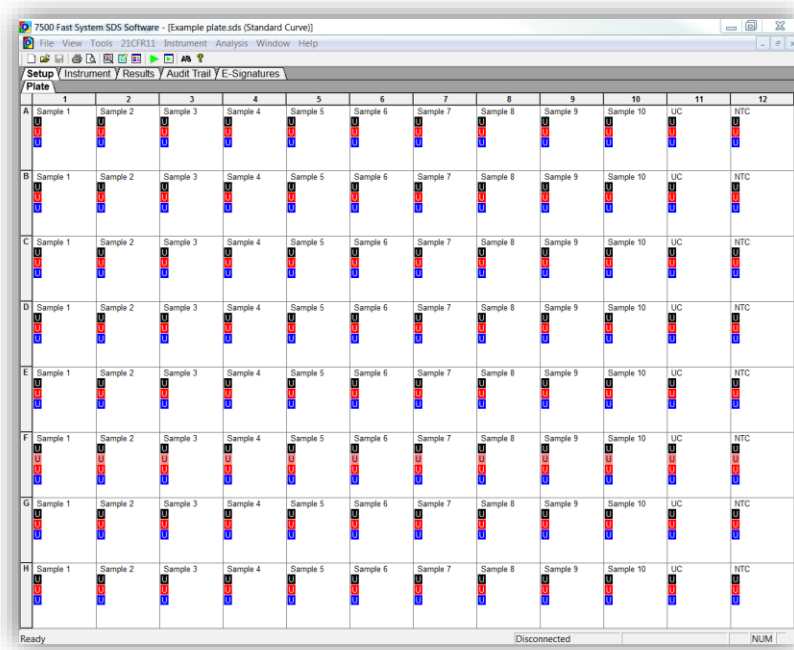
7. Enter the Operator's name, insert lot# in the Comments section and the Plate name e.g. "qPCR II 081214", then click Finish:



8. In the "Setup" tab, insert the names of the samples. To choose a column press the column number, then write the sample name.



9. Repeat step 8 according to the number of samples. **Important:** the control samples should be named “UC” and “NTC” in capital letters.
10. The plate view will be updated according to the sample names:



11. In the “Instrument” tab, verify that the following thermal profile is defined:

Table 31 - PCR Thermal profile

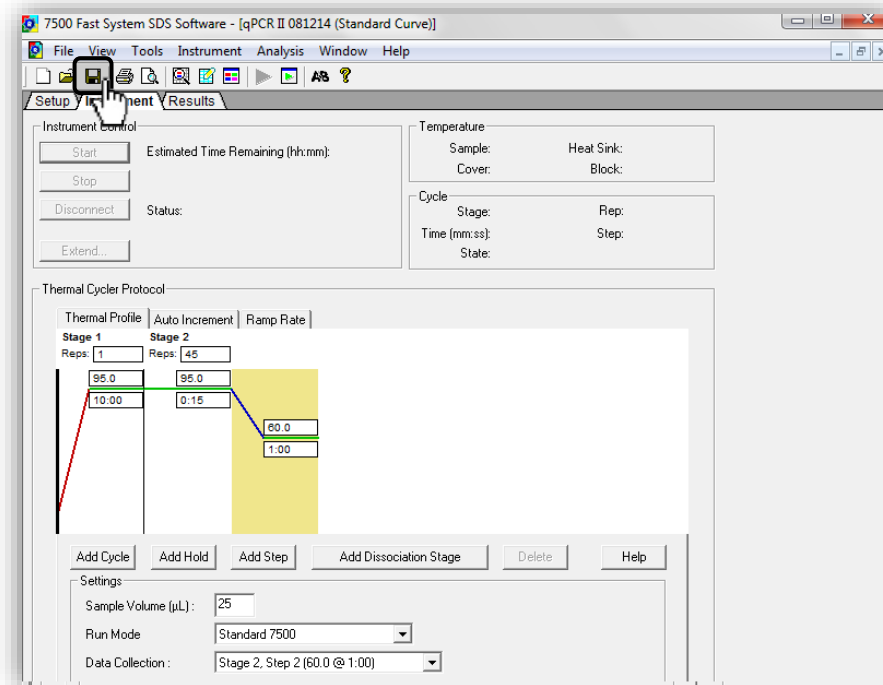
Stage	Temperature (°C)	Duration	Repeats
1	95	10:00 (min)	1
2	95	00:15 (min)	45
	60	01:00 (min)	*detection

Sample volume: 25µl

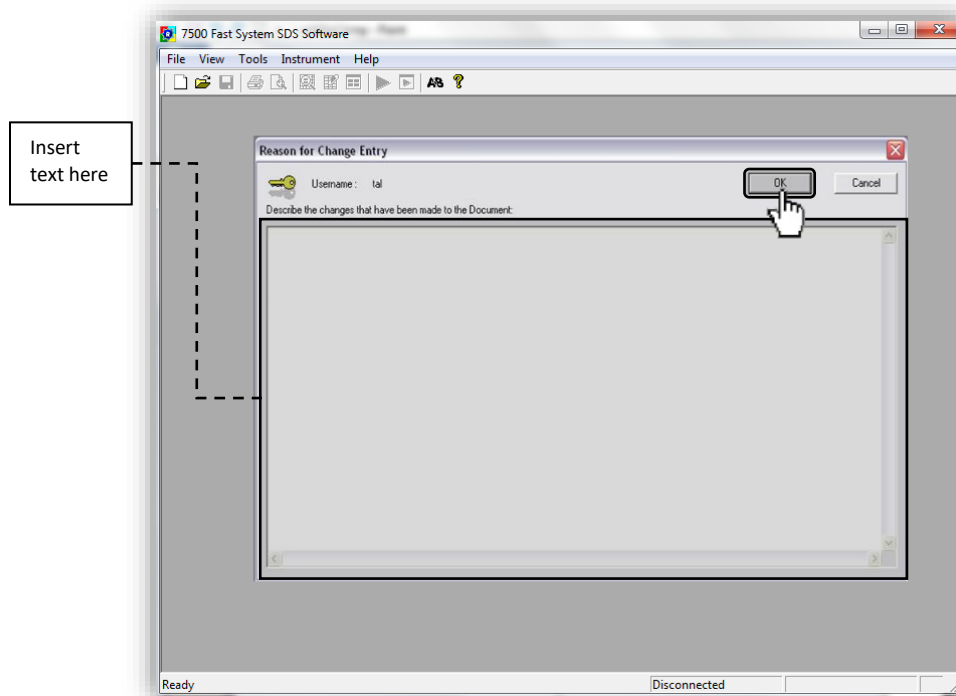
Run Mode: Standard 7500

Data collection: Stage 2, step 2 (60.0 @ 1:00)

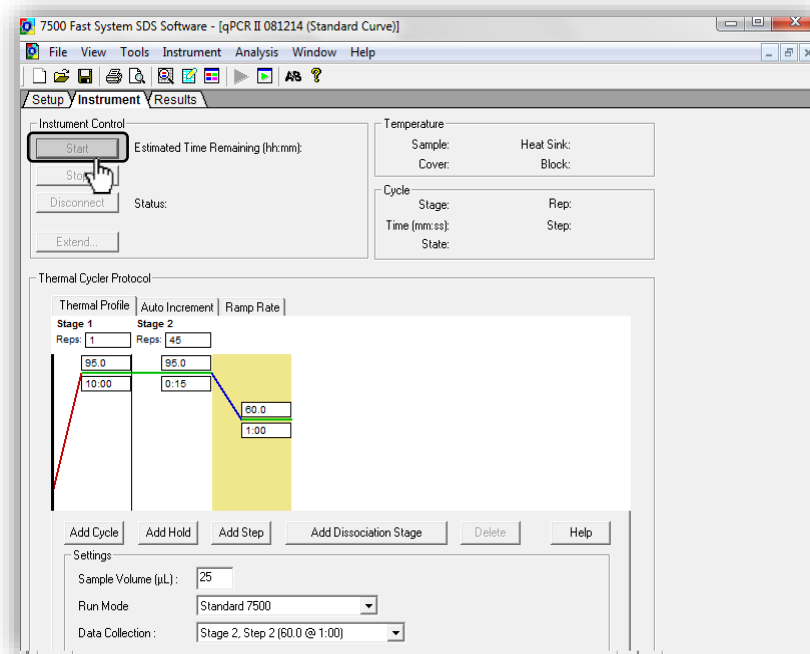
12. In the instrument tab press the "Save" button and give the run a name:



13. The system will then automatically ask for a reason for the changes in the template, write any text in the box, then click "OK".



14. Press the "Start" button:



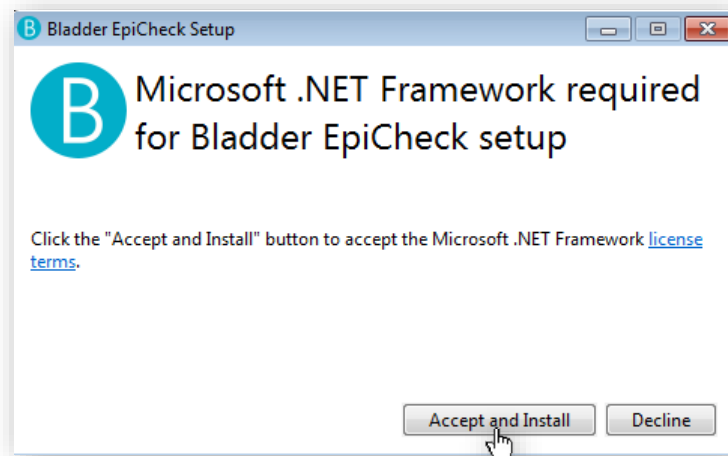
11. Analysis with Bladder EpiCheck Software

11.1. Installation

If due to regulatory reasons it is not allowed to install the software on the device's computer, perform the following steps:

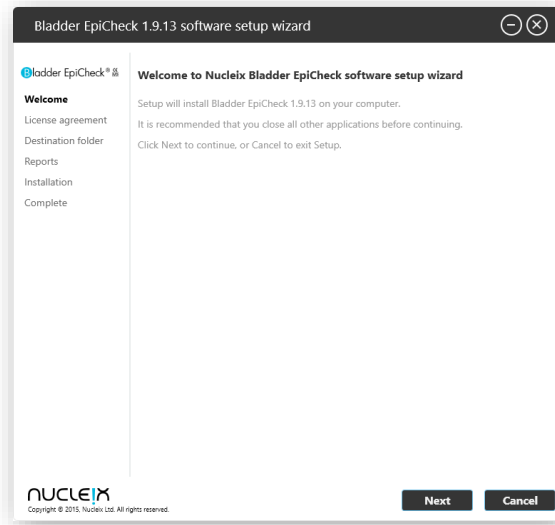
- Install the software on a different computer and use a USB Flash Drive to locate the template.
- Locate the template to the following path:
C:\Applied Biosystems\SDS Documents

- 1) Double click on the "BladderEpiCheck.Setup" icon.
- 2) The Installer will check if your computer has .NET Framework 4.0 and above, sufficient hard drive storage space and sufficient RAM (necessary for the Bladder EpiCheck software). If .NET is installed (.NET is installed on Windows 7 by default) and all other parameters meet the requirements, you will automatically proceed to step 4. Otherwise, one of the following will occur:
 - a. The Bladder EpiCheck installer will install .NET Framework 4.0.



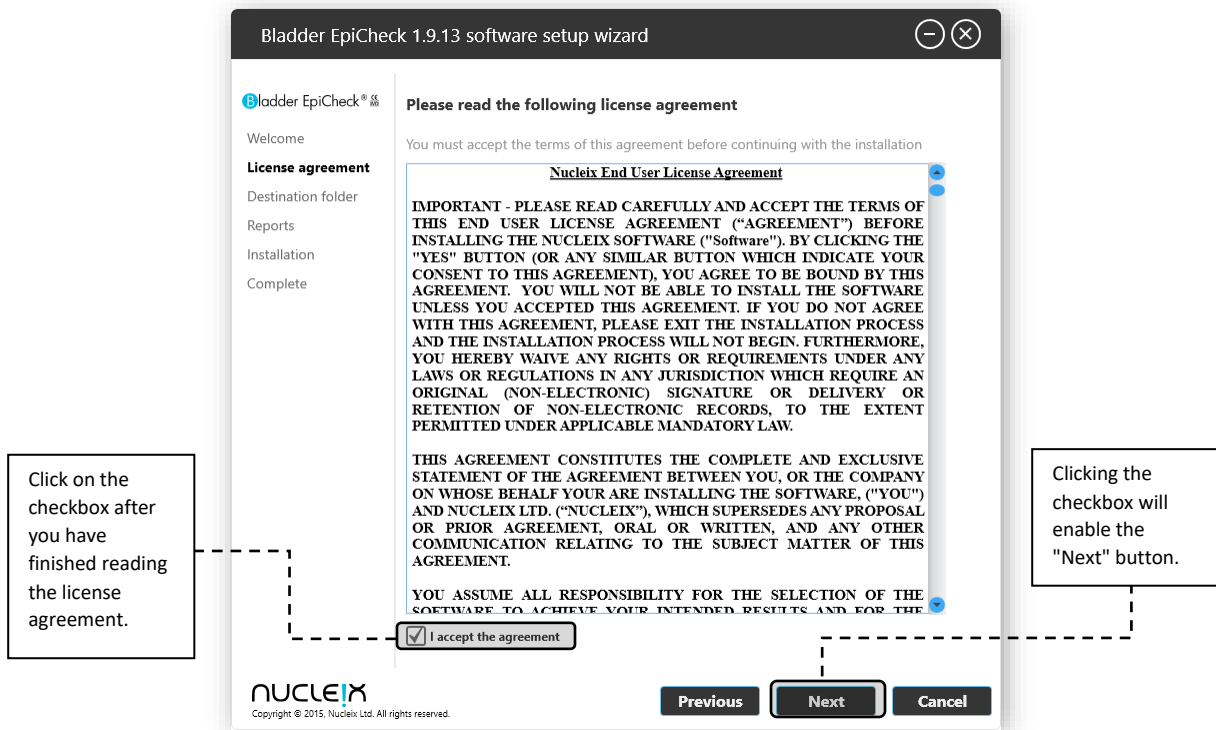
- b. An error will inform that the computer has insufficient hard drive storage space and/or RAM.

- 3) After .NET is successfully installed, the Bladder EpiCheck software setup wizard will pop up.



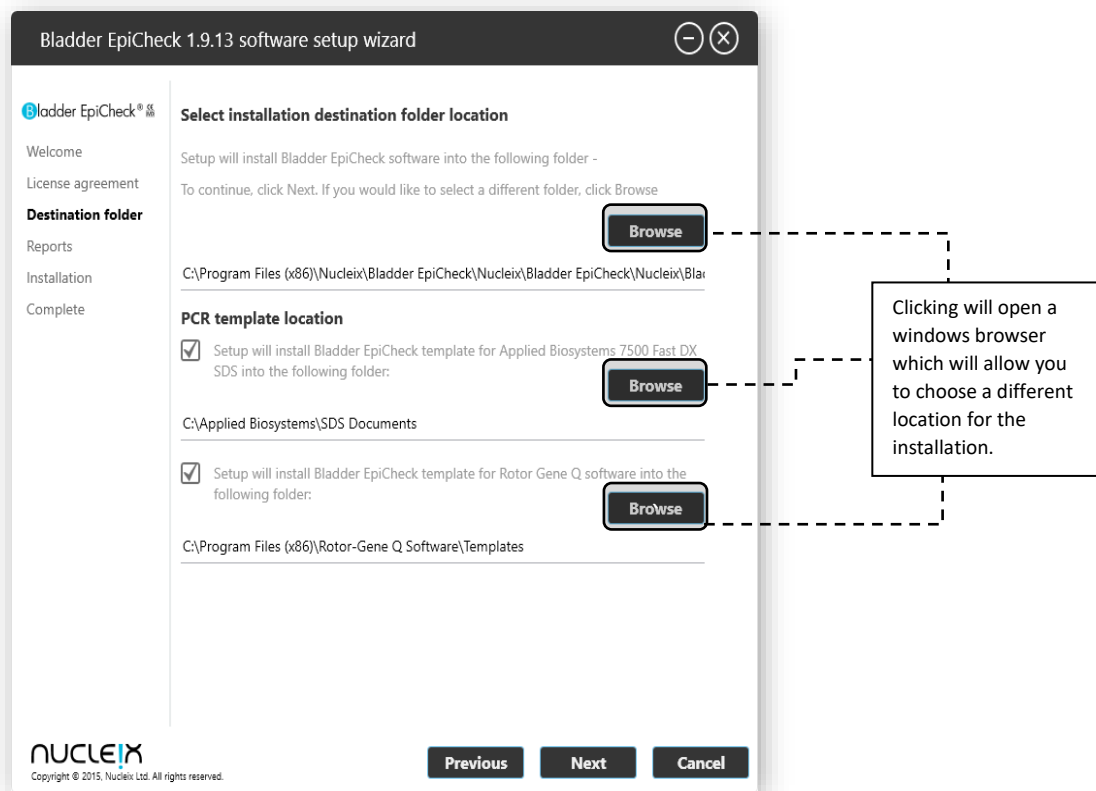
- 4) On the left side of the setup window you can see the current installation procedure step and the remaining steps. Click "Next" to continue with the installation.

- 5) In the "License agreement" step you are required to read the license agreement. Agreeing to the license agreement is mandatory for continuing with the installation.



- 6) Click "Next" after clicking the checkbox.
- 7) In the "Destination folder" step you can choose the following:
 - a. Bladder EpiCheck software location. The default location is the "Program Files (x86)" folder.
 - b. PCR template location for Rotor Gene Q software and for ABI 7500 fast Dx software.

In order to change the location, click on the "Browse" and choose the desired location



- 8) In the "Reports" step you can choose where the Bladder EpiCheck software will automatically generate both patient and summary reports. Also, you can insert your laboratory details and logo which will be added to the reports. The default location is in shared documents (C:\Users\Public\Documents).

Bladder EpiCheck 1.9.13 software setup wizard

Bladder EpiCheck®

Welcome
License agreement
Destination folder
Reports
Installation
Complete

Reports settings

The Bladder EpiCheck software run includes reports and analyses generation. In order to proceed with the installation please fill in the following information.

Please specify the location for report output files:
C:\Users\Public\Documents\Bladder EpiCheck\Reports **Browse**

Please specify the location for analysis output files:
C:\Users\Public\Documents\Bladder EpiCheck\Analysis **Browse**

Reports language: English (United States)

Laboratory details for report

Name: _____

Address: _____

Phone number: _____

Email: _____

Logo: _____ **Browse**

Disclaimer: _____

NUCLEIX
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Previous **Next** **Cancel**

Clicking will open a windows browser which will allow you to choose a different location for the reports.

Clicking will open a windows browser which will allow you to choose a logo.

- 9) Click "Next" to initiate the installation, and wait for the installation to complete.

Bladder EpiCheck software setup wizard

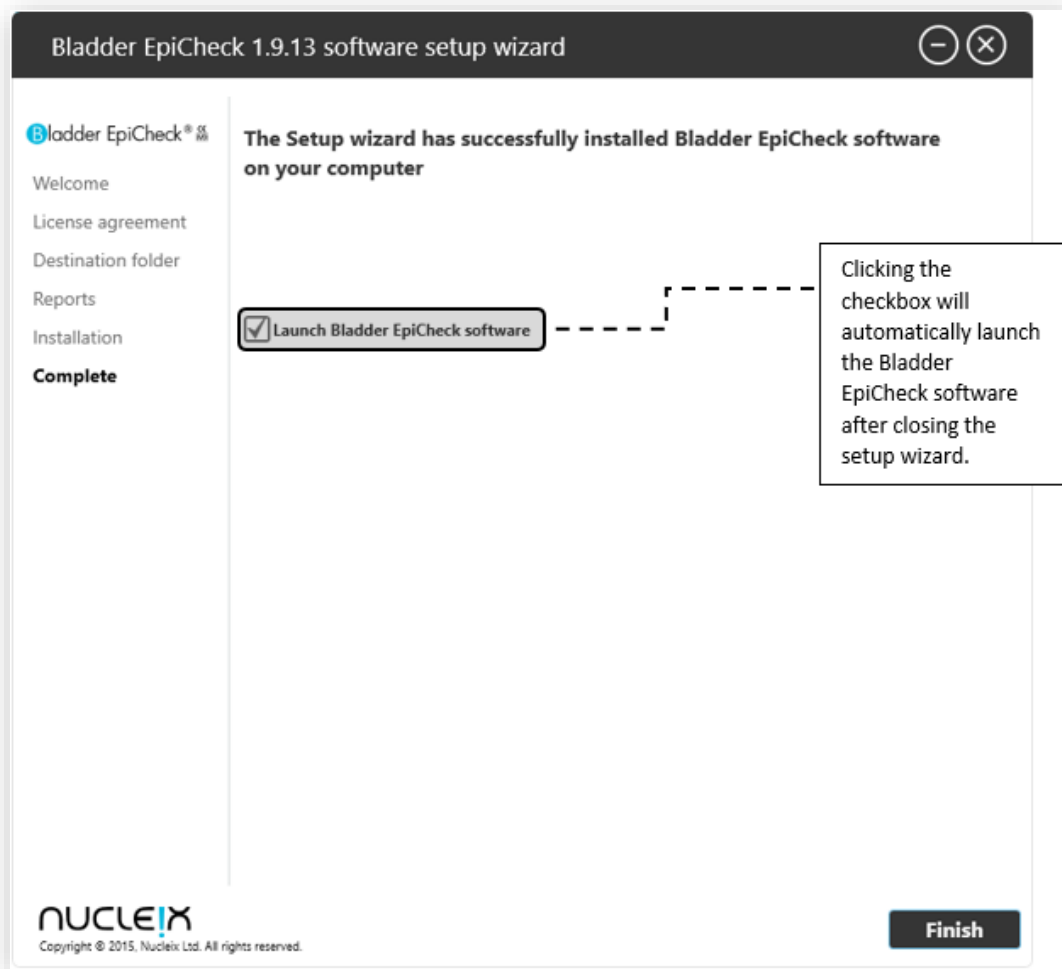
Bladder EpiCheck

Welcome
License agreement
Destination folder
Reports
Installation
Complete

NUCLEIX
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Cancel

10) The wizard will notify you that the installation has been completed successfully:



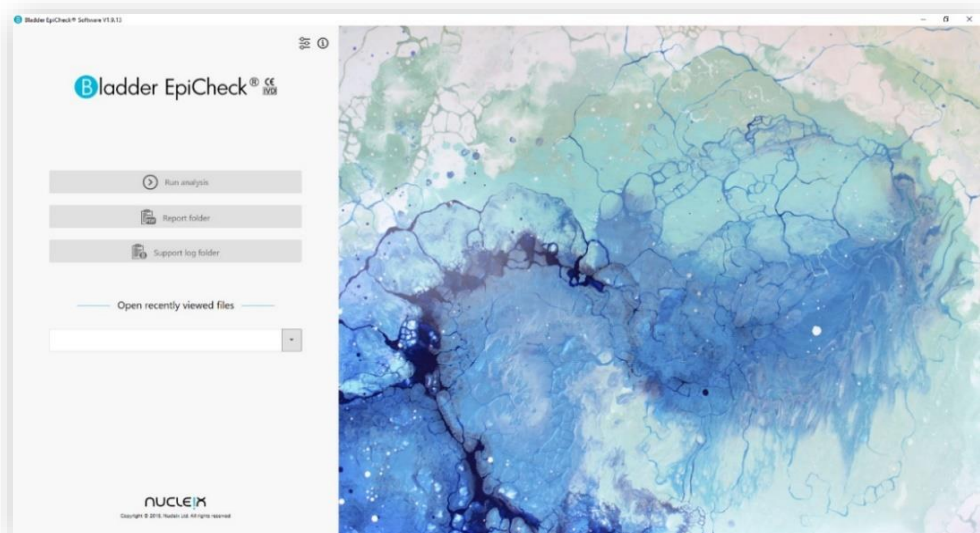
11) Exit the setup wizard by clicking "Finish" or closing the setup window.

Note:

If you are trying to reinstall Bladder EpiCheck while the software is running, an error will inform you that the software must be closed to allow setup to continue. By pressing "OK", the software will be closed.

11.2. Getting started

After launching the software, the quick startup screen is the first to appear. From the startup page you can load your desired real-time PCR file (for ABI 7500 Fast Dx the file type is. sds). To learn more on how to load a file refer to the section 11.4 “Loading a real time PCR file” ([page 55](#)).

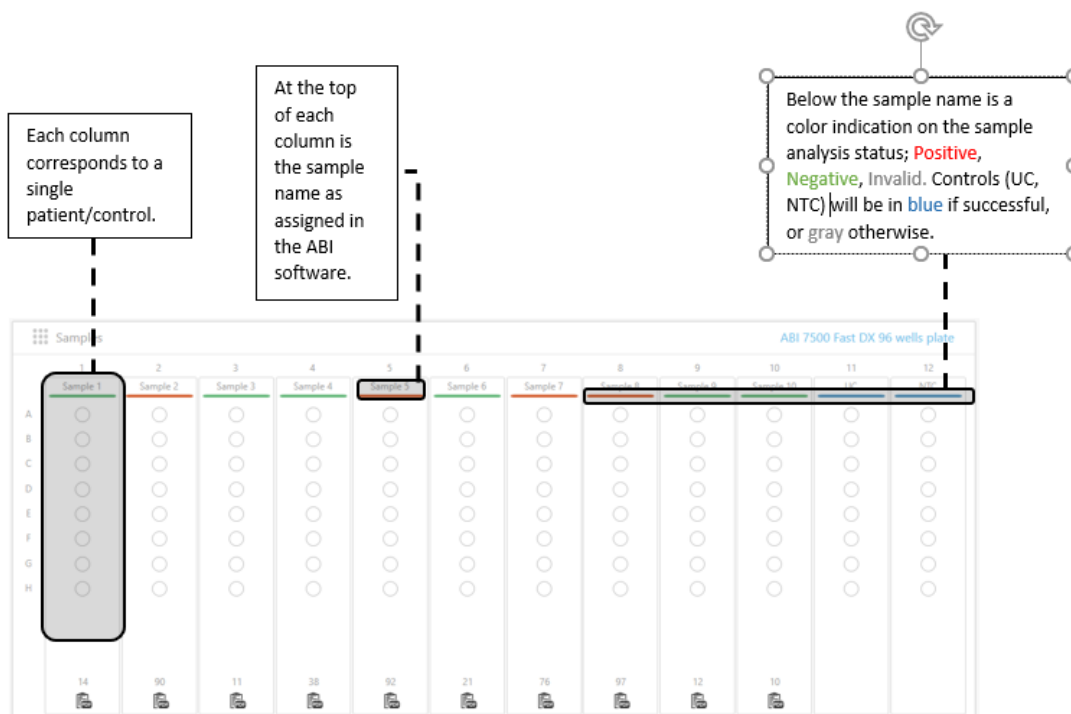
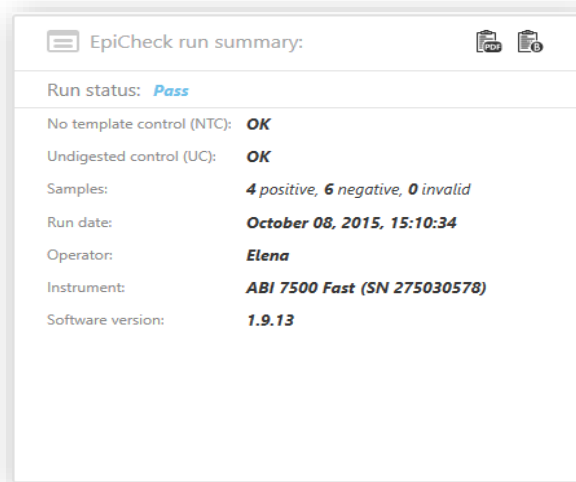


The Bladder EpiCheck software automatically analyzes the files once they are loaded into the software.

See below to get a basic understanding of the software.



In the summary box you can find basic information regarding the analysis.



11.3. Explanation of the analysis

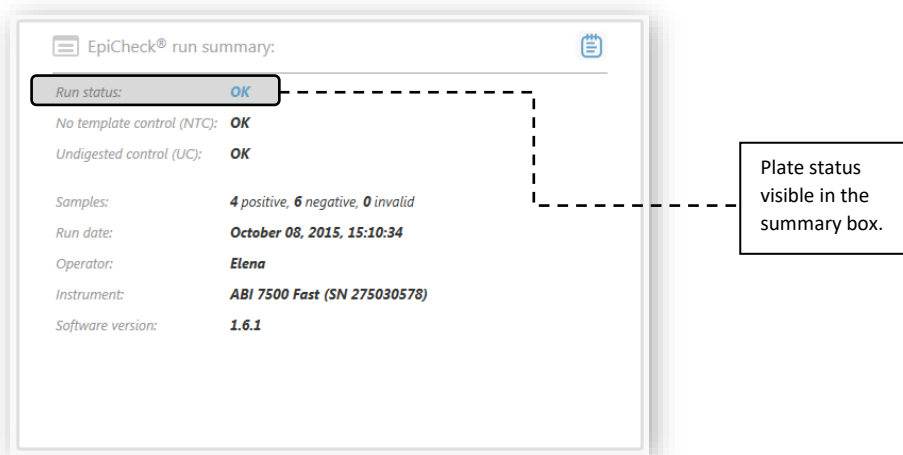
Overview

The Bladder EpiCheck software automatically analyzes loaded real-time PCR files. Analysis is performed in two steps: (i) EpiCheck run analysis and (ii) sample analysis. First the software determines whether the EpiCheck run status is OK or not. If the run status is not OK, the

software will not continue to analyze the samples and they will all receive the Invalid analysis. If the EpiCheck run status is OK, then the software will proceed to analyze each sample. For each sample, the software first checks whether its controls are OK. If one or more of the sample controls are not OK, the sample will receive the Invalid analysis. If all sample controls are OK, the software calculates the sample EpiScore – a number between 0 and 100 representing the overall methylation level of the sample at the panel biomarkers. If the EpiScore is equal or above 60 then the patient is defined to be Positive (indicating a high risk for bladder cancer or upper tract urothelial carcinoma (UTUC)), otherwise the patient is defined to be Negative (indicating a low risk for bladder cancer or UTUC).

EpiCheck run analysis

The status of the EpiCheck run and of the two control samples are shown in the summary box:



Possible reasons for EpiCheck run failure:

- UC sample failed
- NTC sample contaminated
- Missing UC sample
- Missing NTC sample
- Too Many UC samples
- Too Many NTC samples

Note: If you received any of the above errors see Section 12 - Troubleshooting ([page 68](#)).

Sample analysis

If the EpiCheck run status is OK the software will proceed to analyze each of the patient samples.

In order for a sample to be analyzed the following criteria need to be met:

- All sample wells have the same (non-empty) sample name, as entered in the ABI 7500 Fast Dx software
- Sample digestion was successful
- There was a sufficient amount of DNA in all sample wells

If these criteria are met, then the sample will be diagnosed either as Positive or Negative. Otherwise, the sample will not be analyzed and the sample analysis will be Invalid.

Note: If you received any of the above errors see Section 12 - Troubleshooting ([page 68](#)).

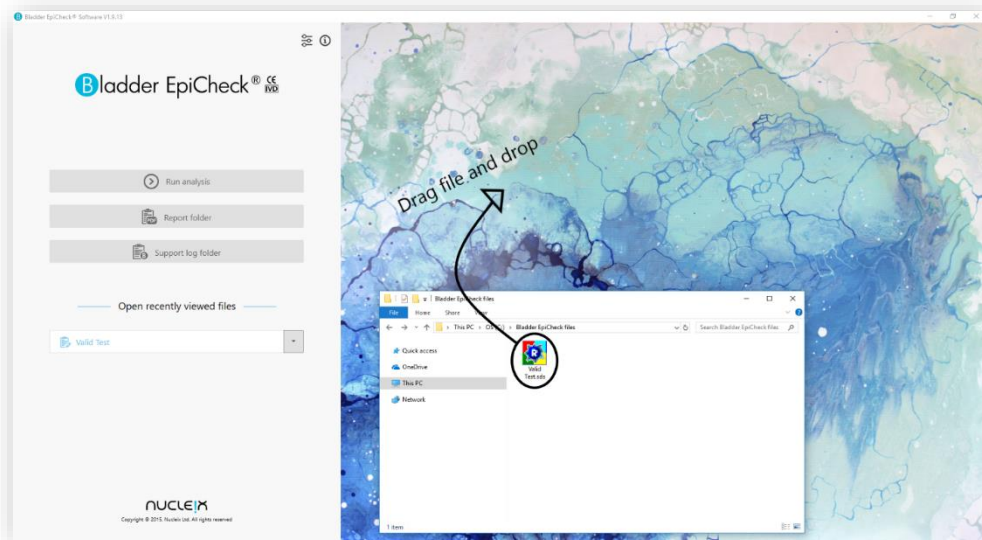
11.4. Basic functions

Loading a real time PCR file

There are 4 ways in which you can load real time PCR file into the Bladder EpiCheck software for analysis:

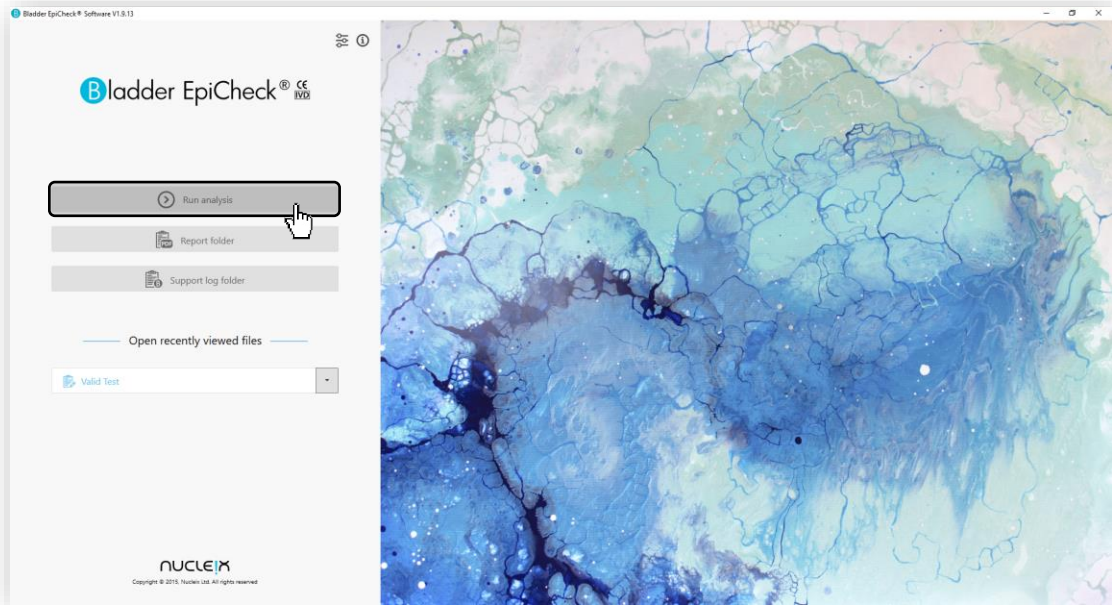
(1) Drag and drop

1. Launch the Bladder EpiCheck software
2. Locate the file for analysis using windows explorer (for ABI 7500 fast DX the file type is .sds)
3. Drag and drop the desired file in the Bladder EpiCheck software window

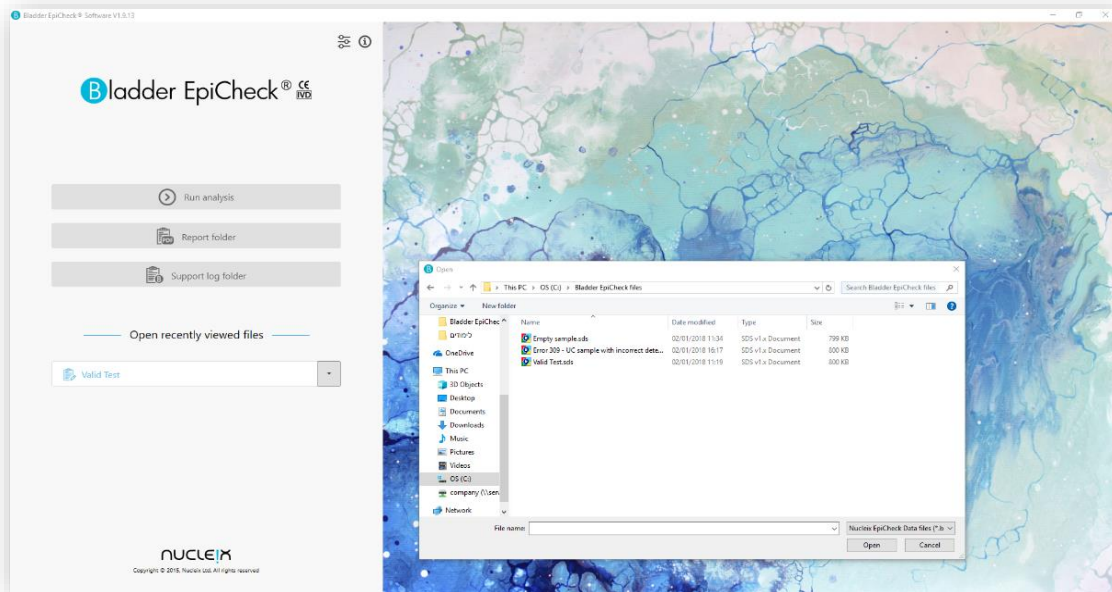


(2) Analyze new file button

1. Open the Bladder EpiCheck software
2. Click on the "Run analysis" button/icon

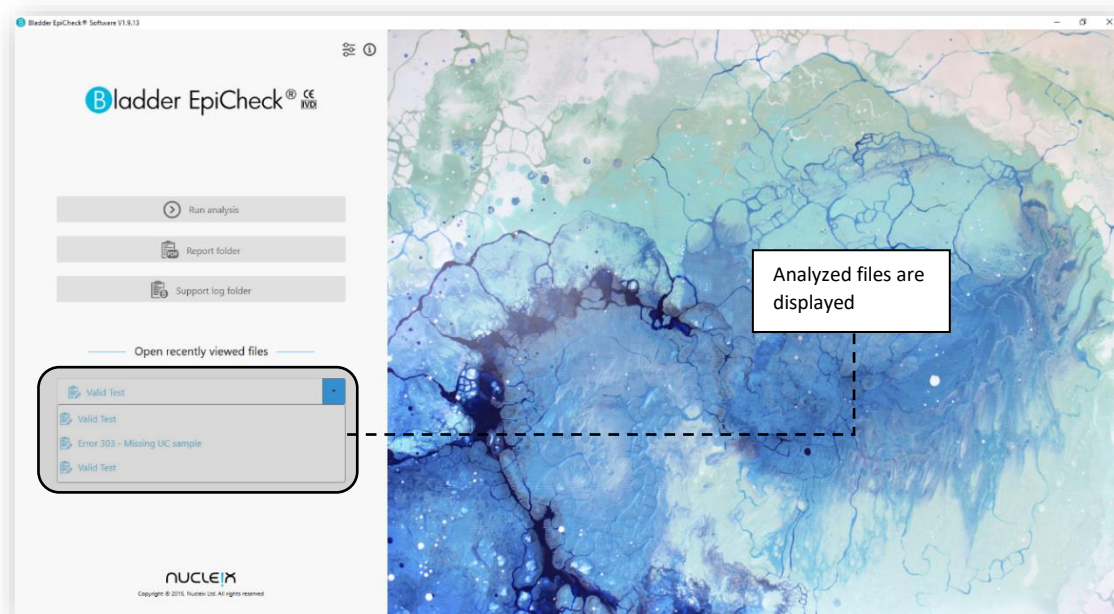


3. Select the file from the windows browser and click open/double-click the file.



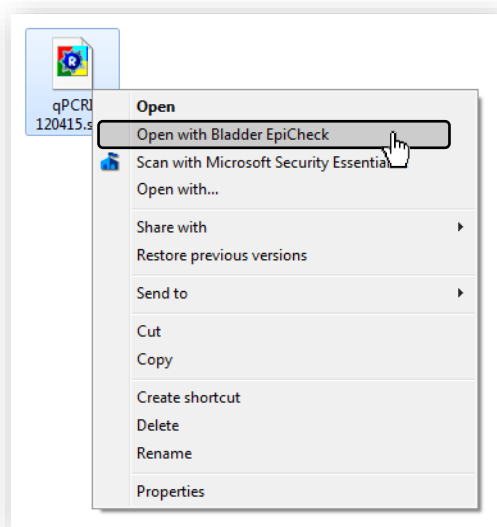
(3) Recent analysis

The Bladder EpiCheck software allows you to easily reload recently analyzed files. When launching the software, on the Quick startup page, you can see the 3 files that were most recently analyzed. Click a file icon to reload it into the Bladder EpiCheck software.

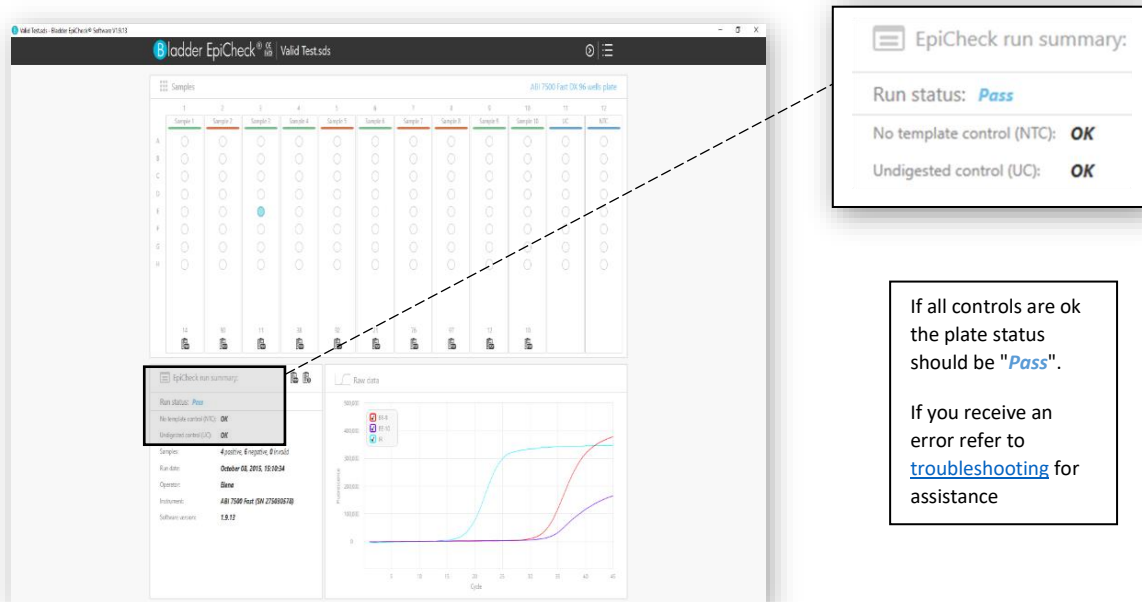
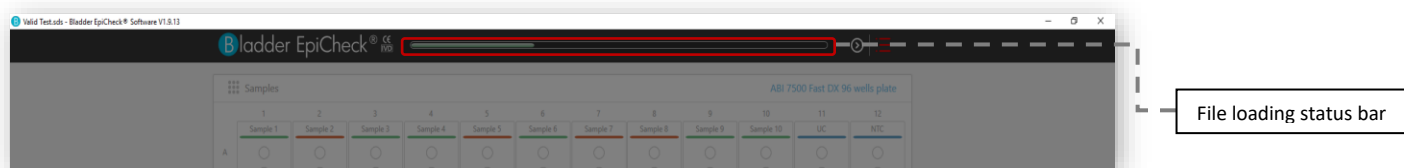


(4) "Open with"

Locate a real-time PCR file and right-click the file. Choose "Open with Bladder EpiCheck" to open the file in the Bladder EpiCheck software.

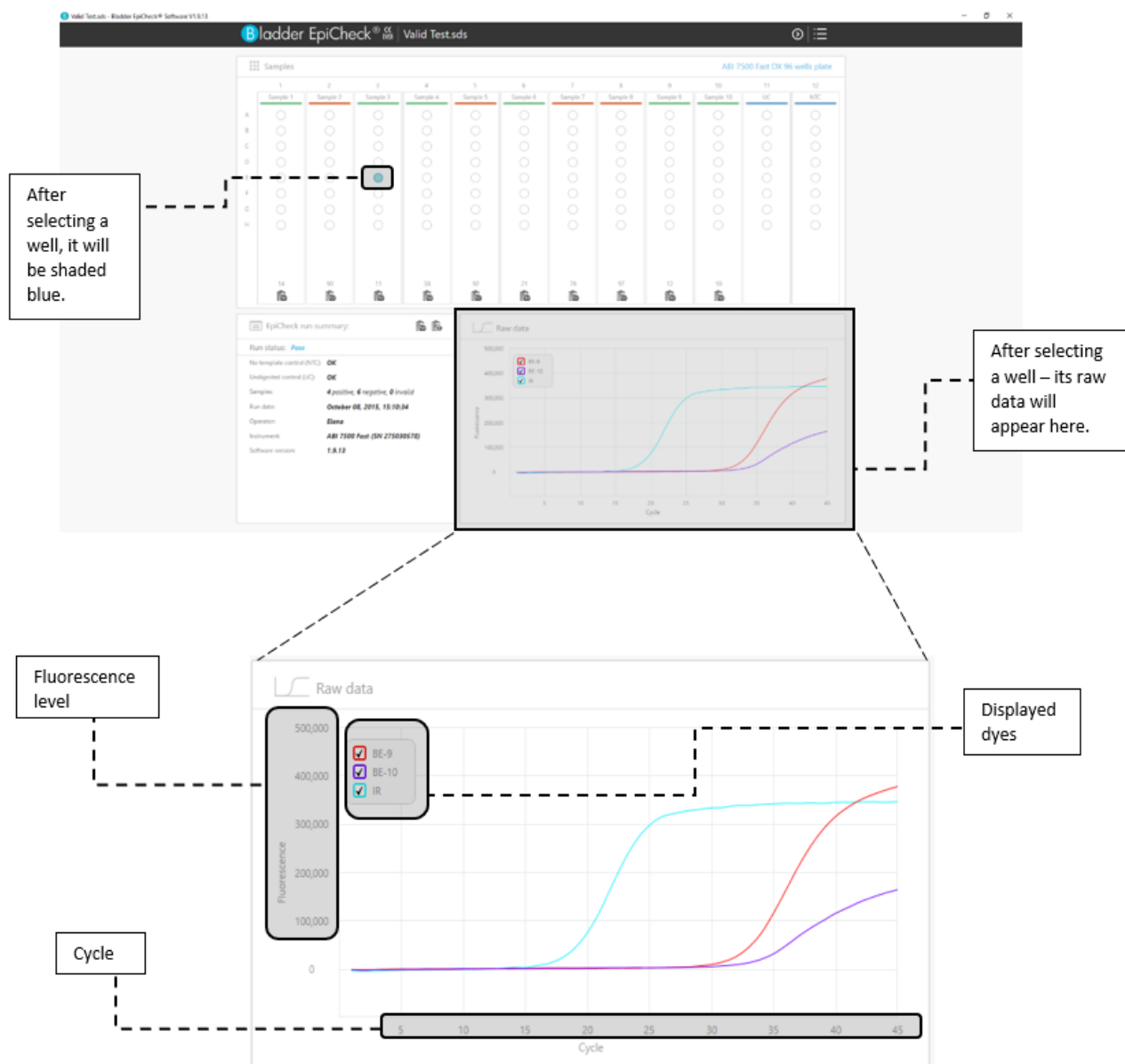


After loading your file using any of these methods an analysis progress bar will appear:

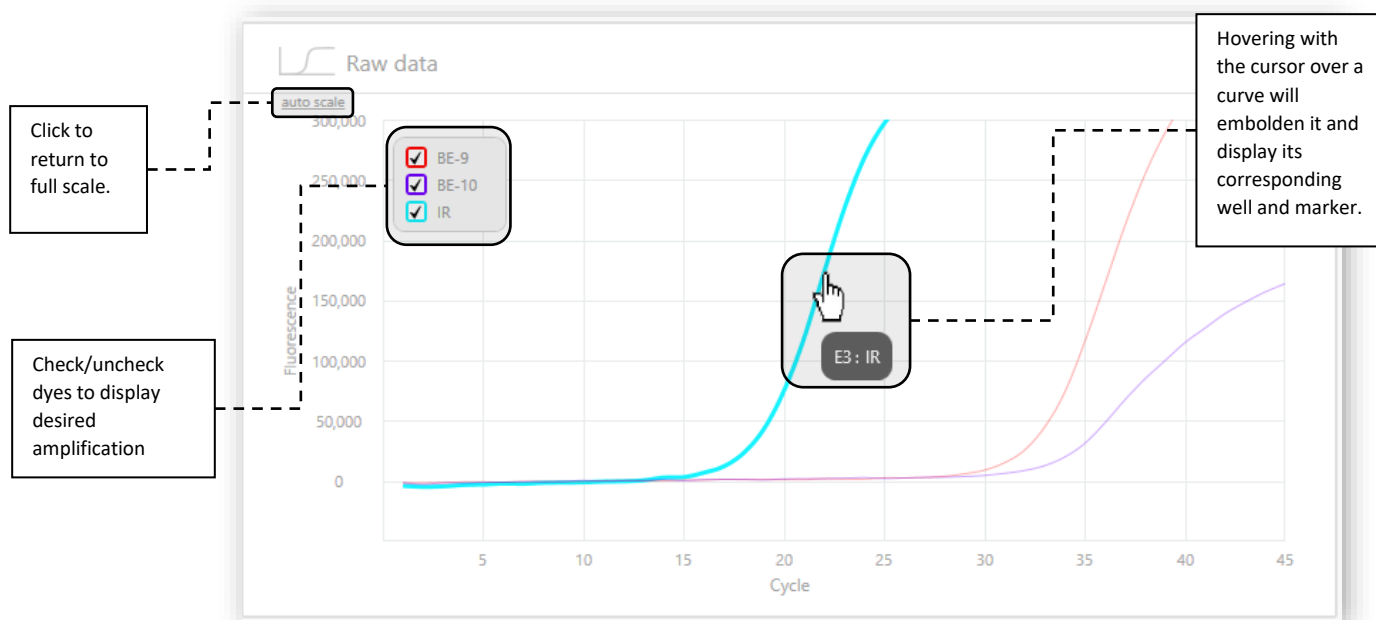
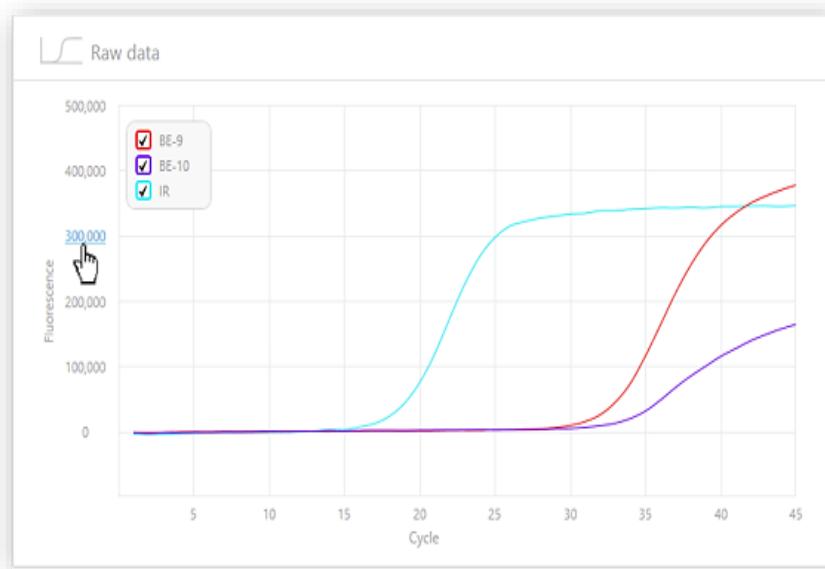


Displaying raw data of a single well or multiple wells

In order to view the raw data (the amplification curves) of a single well click on the desired well.

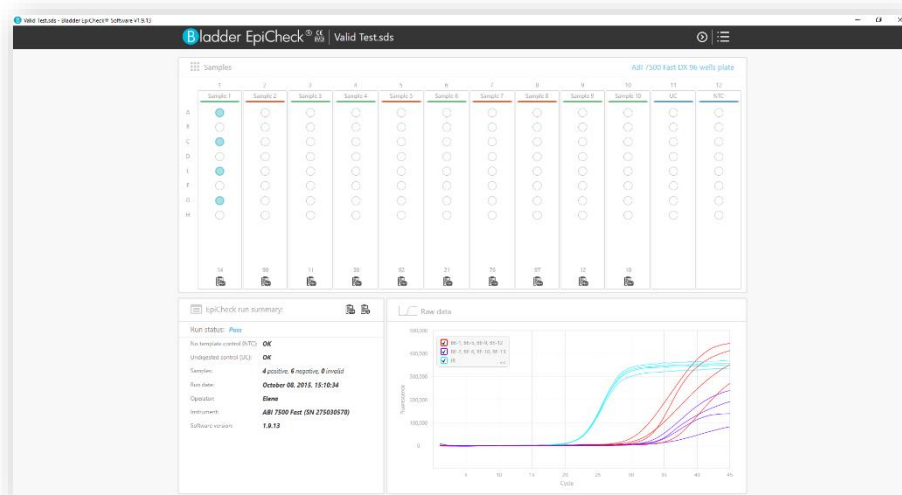


In order to zoom on the Y axis, click on any of the fluorescence level values and the chart will re-scale such that the selected value will be the highest Y axis value. In order to return to full scale, click on "auto scale", located at the top of the chart.



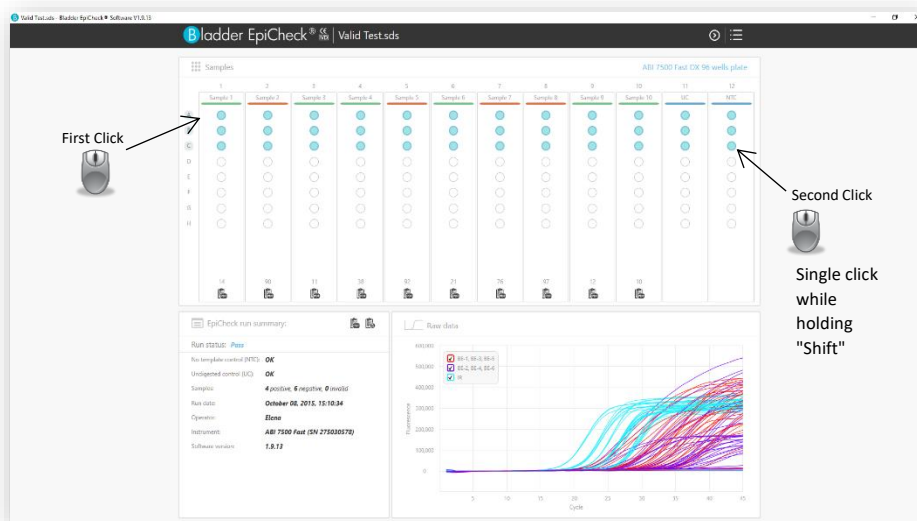
Below are various ways to view multiple wells:

Selecting multiple wells using "Ctrl"

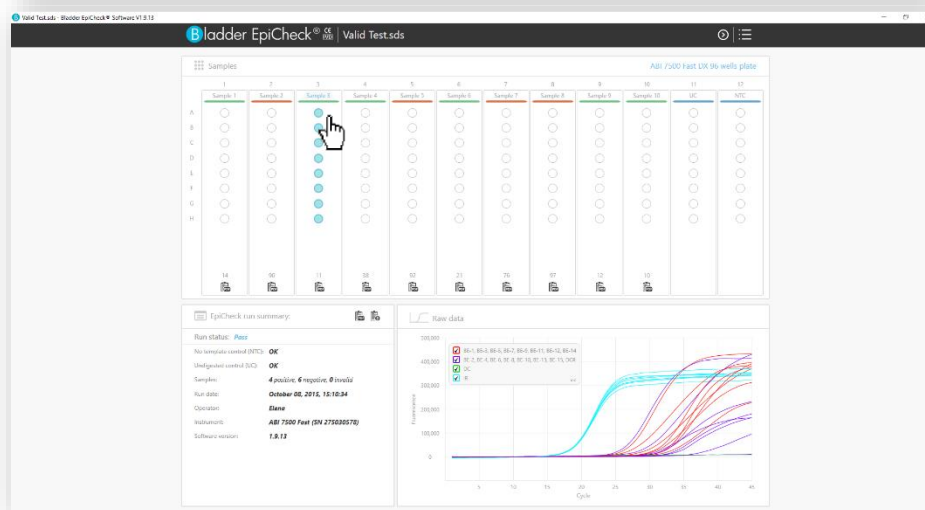


Graph will display in overlay the raw data of all selected wells and dyes.

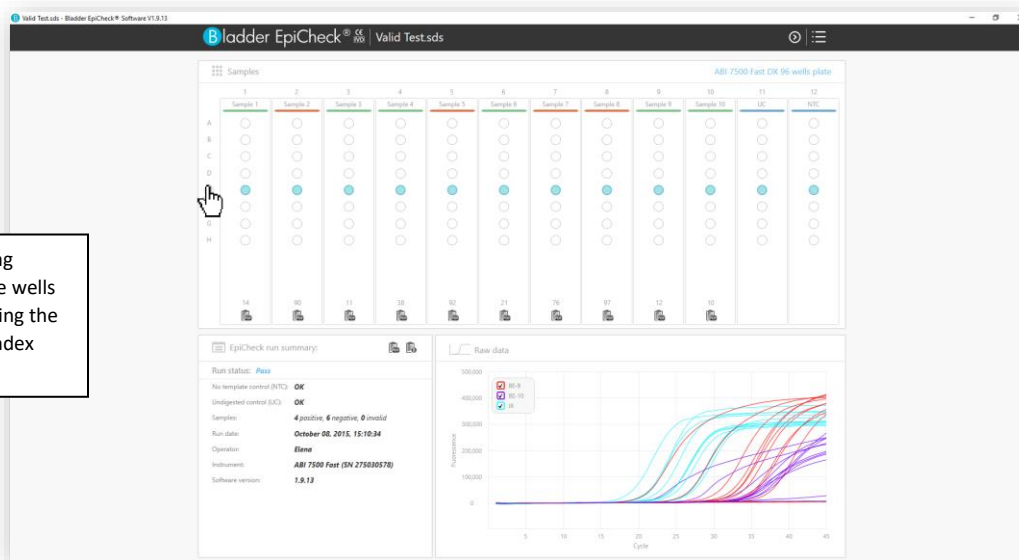
Selecting multiple wells using "Shift"



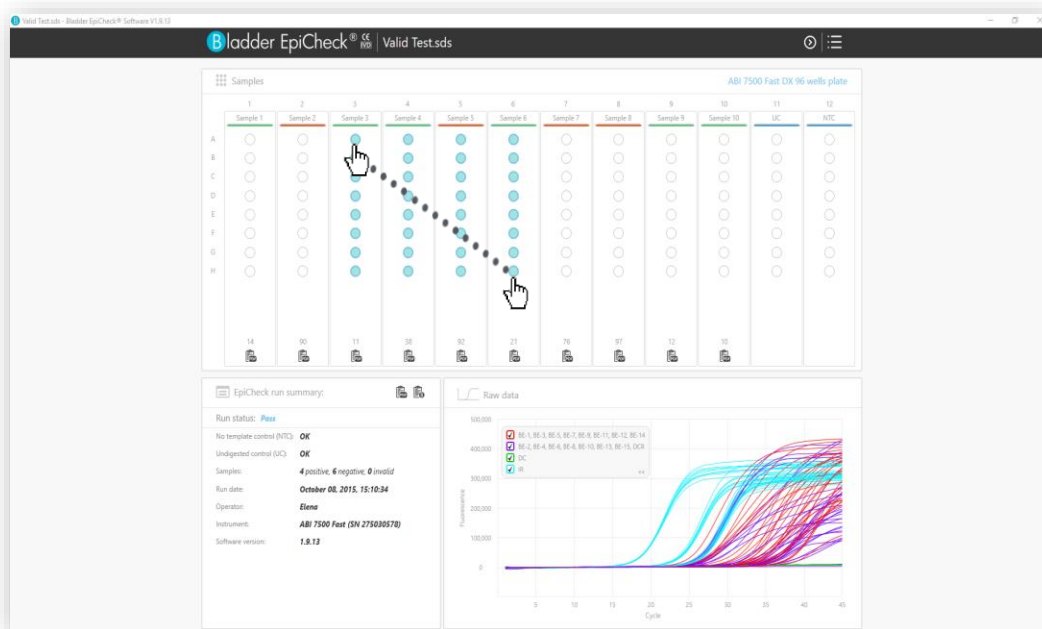
Selecting multiple wells by clicking the sample name



Selecting multiple wells by clicking the row's index letter



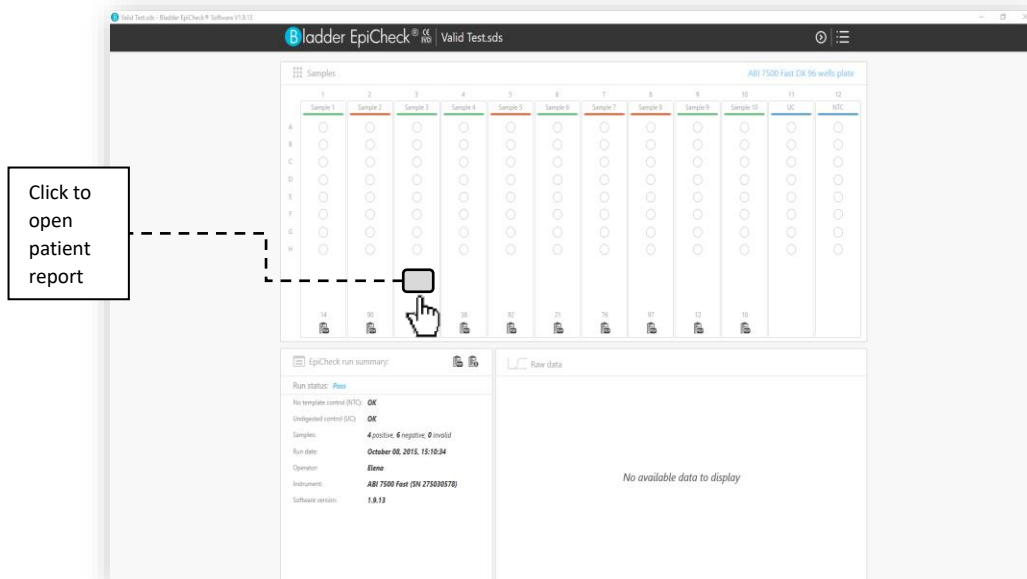
Mouse drag selection



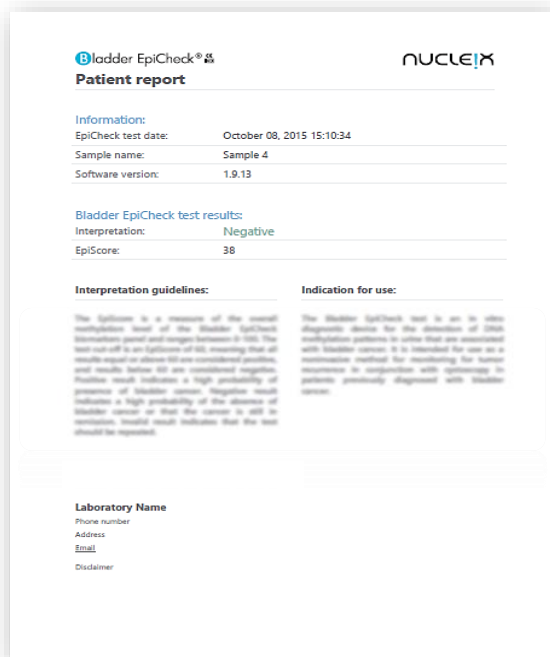
Opening a patient report

The Bladder EpiCheck software automatically creates a report for each patient sample (.pdf file). The report will be generated regardless of whether analysis of the sample was successful or not. Reports are not generated for control samples (UC / NTC), and their status can be seen in the summary window.

In order to open a patient's report, click on report logo  located below the desired sample.



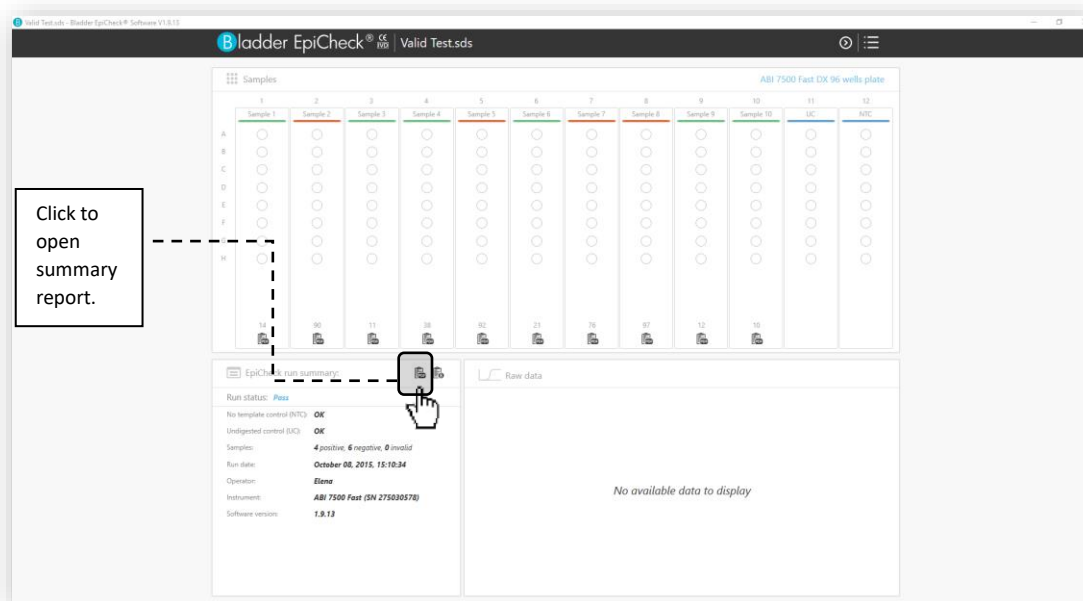
After clicking the report icon, a .pdf file containing the patient's report will be opened:



Under "**Information**" you can find basic information such as sample name and the date of the test. Under "**Bladder EpiCheck test results**" you can find the analysis (Positive/Negative/Invalid) and the EpiScore. In addition, the interpretation guidelines and indications for use are written.

Opening the laboratory summary report

The Bladder EpiCheck software automatically creates a laboratory summary report including results of all samples as well as information of the PCR run (a .pdf file).

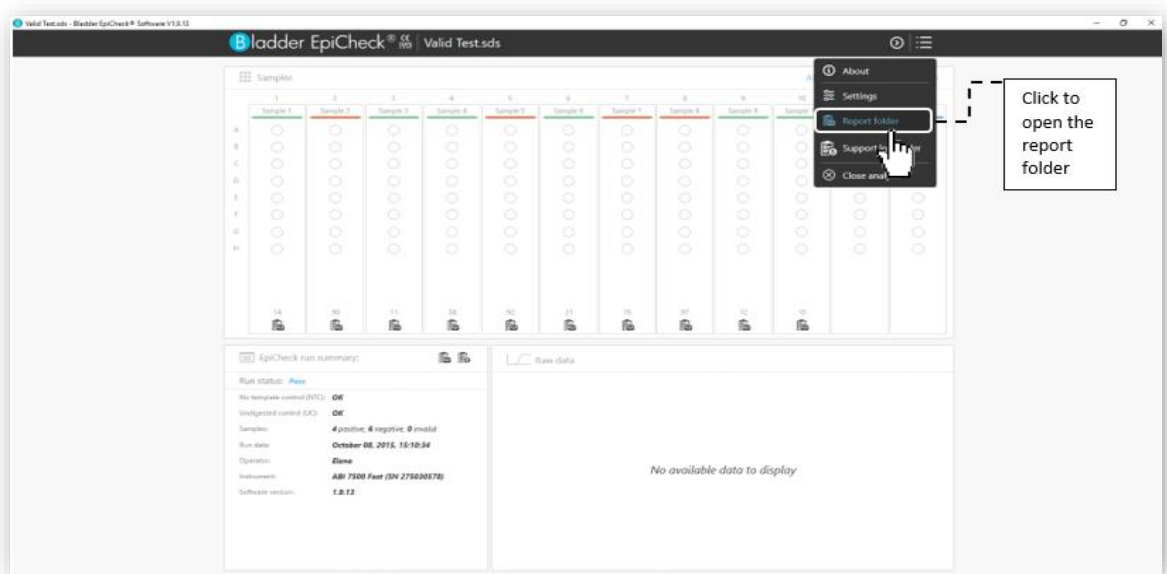


After clicking the report icon, a .pdf file containing the Run summary report will be opened:



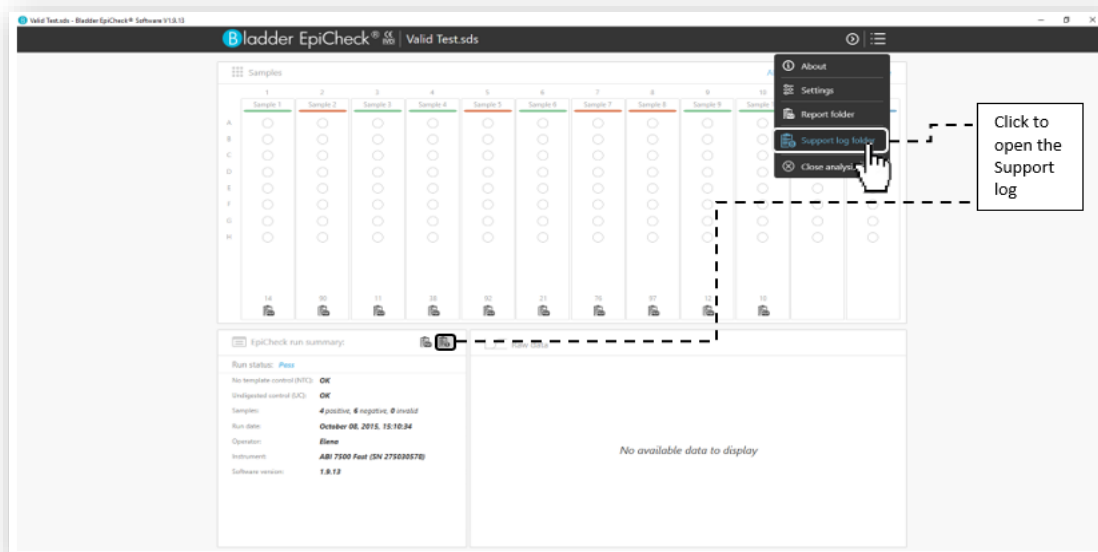
Open report folder

The report folder is the folder in which the Bladder EpiCheck software saves all the Patients report files and the Run summary report for the current EpiCheck run. In order to view the report folder click the menu icon (☰) located on the top right of the application window and click the "Report folder" tab.



Open Support log

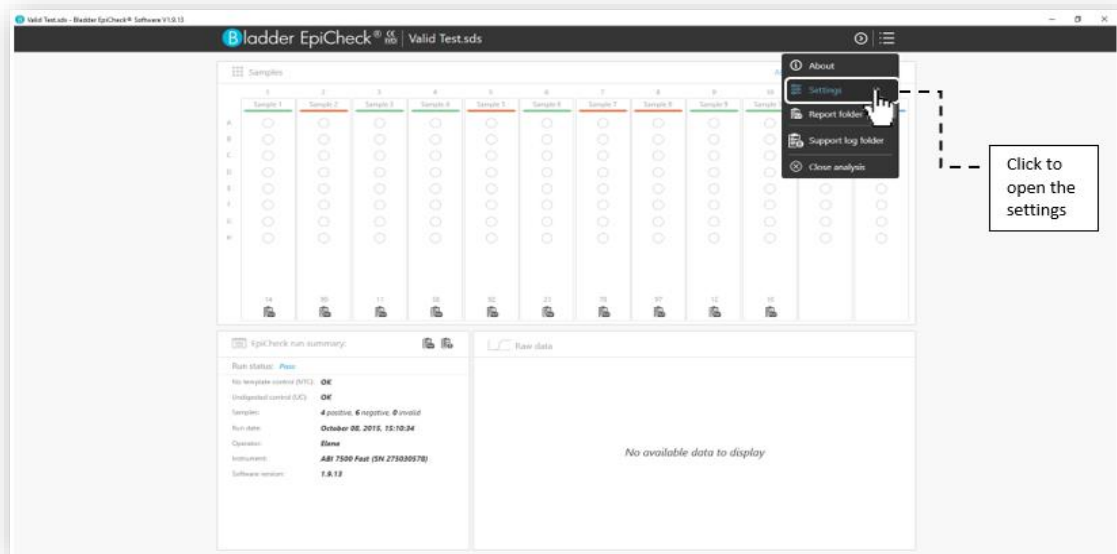
The support log folder is the folder in which the Bladder EpiCheck software saves a .becp file. In order to view the report folder click the menu icon (☰) located on the top right corner of the application window and then click the "Support log folder" tab. In addition, you may open the Support log folder by clicking the Summary report icon.



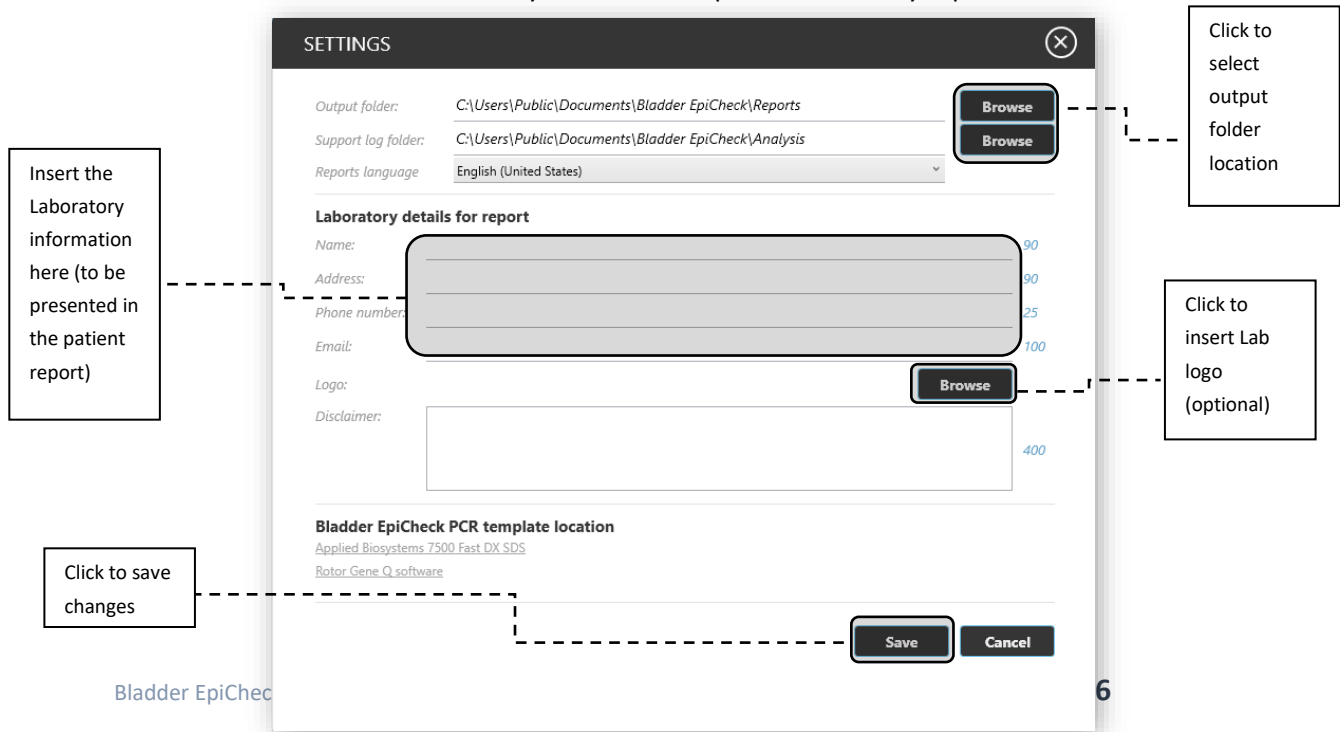
Update settings

In the "Settings" menu () you can change the following:

- Output folder location for automatically generated patient and summary reports.
- Laboratory details presented in the patient report.
- Bladder EpiCheck PCR templates location



In order to change the output folder location, click the "Browse" button and choose the location in which the software automatically saves the sample and summary reports.



11.5. Limitations of the Procedure

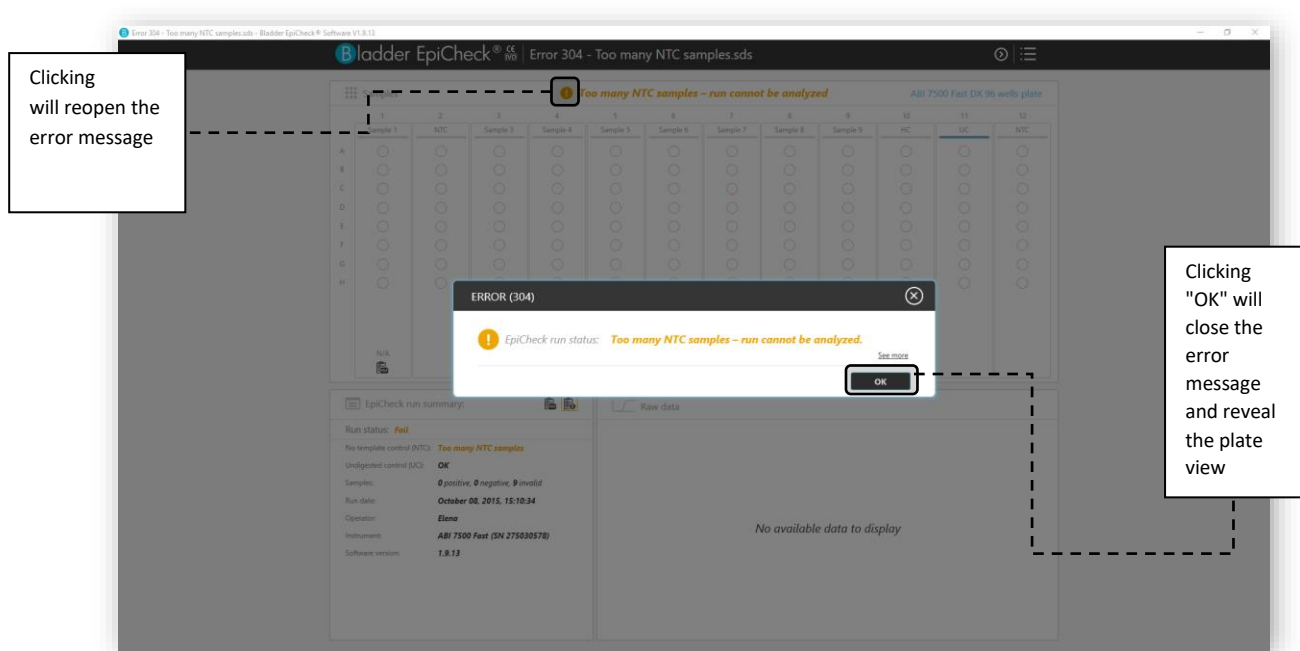
- For in vitro diagnostic procedures.
- Use of this product is limited to trained laboratory personnel.
- The Bladder EpiCheck test has been validated with the following only, use of other methods has not been validated:
 - DNA extracted using the Bladder EpiCheck DNA Extraction Kit
 - Digestion and PCR using the Bladder EpiCheck Test Kit
 - Real-time PCR instruments: Applied Biosystems (ABI) 7500 Fast Dx instrument with the Sequence Detection Software version 1.4, or QIAGEN Rotor Gene Q instrument and software
 - Analysis with the Bladder EpiCheck software.
- The Bladder EpiCheck test is aimed as an adjunct to current procedures, and therefore clinical interpretation of test results should be within the context of patient's medical history and in conjugation with other diagnostic test results.
- Analysis of results should be performed using the Bladder EpiCheck software. Positive result indicates a high probability of presence of bladder cancer or upper tract urothelial carcinoma (UTUC). Negative result indicates a high probability of the absence of bladder cancer and UTUC or that the cancer is still in remission. Invalid result indicates that the test should be repeated.
- Reliable results are dependent on correct specimen collection, transportation storage and processing. Please follow the procedure as described in this Instructions for Use.
- The Bladder EpiCheck test is a qualitative test.
- High levels of Uric acid present in urine collected from males may cause an Invalid result in the Bladder EpiCheck Assay due to interference. In case of Invalid result, the test should be repeated with a new urine sample.

12. Troubleshooting Guide

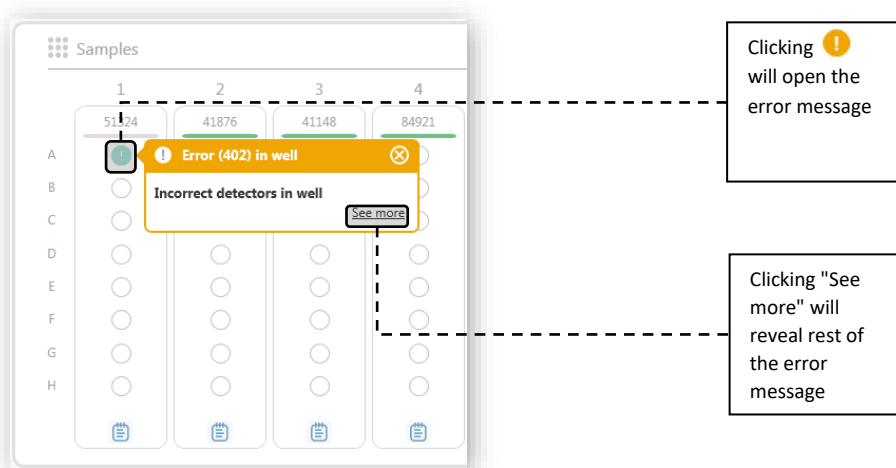
This troubleshooting guide may be helpful in solving any problems that might arise. For more information, see also the Frequently Asked Questions at our Technical Support Center: www.nucleix.com/customer-area/. For any further questions please contact our technical support team at support@nucleix.com.

There are 4 types of errors: (i) Setup errors, (ii) File/Settings errors, (iii) EpiCheck run errors, and (iv) Sample errors.

An example of a plate error:



An example of a sample error:



12.1. Bladder EpiCheck software errors

Table 32 - Setup errors

Error number	Error message	Meaning	Fix suggestion
101	A fatal error occurred! Missing required resources. The application is shutting down. Please reinstall the software.	One of the required resources of the software is missing.	N/A
102	Missing required Settings information. You will be prompted to fill in this information.	Reports output folder location is not defined.	N/A
103	<file name> document cannot be opened because PDF Reader program is not installed or not set to be the default program.	PDF reader is either not installed or not set to be the default reader for PDF files.	N/A

Table 32 (continued)

Error number	Error message	Meaning	Fix suggestion
105	A fatal error occurred! Software files missing or corrupted. Application is shutting down. Please reinstall the software.	There might be a problem with one or more files of the EpiCheck software	N/A
106	Insufficient Hard Drive Storage Space The hard drive memory required to store the software analysis is above the currently available storage on this computer. Please create more space on the hard drive and reload the PCR file.	N/A	N/A
107	Insufficient Random Access Memory (RAM) The computer does not have enough RAM to run the analysis. Please verify that the computer has sufficient RAM and reload the PCR file again.	N/A	N/A

Table 33 - File/Settings errors

Error number	Error message	Meaning	Fix suggestion
200	File <filename> is invalid and cannot be loaded. Please verify you load a valid file.	Not a valid real-time PCR file.	N/A
201	An invalid number of PCR cycles (<cycle number> instead of 45). Repeat PCR (see "Protocol for repeating PCR", in the user manual).	PCR program did not perform the expected number of cycles.	Repeat PCR (see "Protocol for repeating PCR" page 77).

Table 33 (continued)

Error number	Error message	Meaning	Fix suggestion
202	File <filename> is not a Bladder EpiCheck run and cannot be loaded. Make sure that the correct file was loaded.	The loaded real-time PCR file isn't a Bladder EpiCheck run and cannot be analyzed.	N/A
203	File < filename > cannot be opened because some of its reports are currently open. Close all reports associated with this file and then press retry.	One of the .pdf report files of the run file are open, thus the file cannot be loaded.	N/A

Table 34 – EpiCheck run errors

Error number	Error message	Meaning	Fix suggestion
300	NTC sample contaminated – run cannot be analyzed.	PCR run invalid – No template control sample was contaminated.	Repeat PCR (see "Protocol for repeating PCR" page 77).
301	UC sample failed in markers <marker list> - run cannot be analyzed.	PCR run invalid – one or more of the markers did not amplify properly in the UC sample.	Repeat PCR (see "Protocol for repeating PCR" page 77). Please contact the Nucleix Support Team for assistance with this issue at: support@nucleix.com.
302	Missing NTC sample – run cannot be analyzed.	In Plate setup in the ABI software none of the samples was named "NTC". Exactly one sample must be named "NTC" for plate to be analyzed.	If you performed the PCR <i>with</i> an NTC sample then open the real-time PCR file in the real-time PCR software, rename the NTC sample wells "NTC" and reload in the Bladder EpiCheck software. If you performed the PCR run <i>without</i> an NTC sample repeat the PCR, (see "Protocol for repeating PCR" page 77).

Table 34 (continued)

Error number	Error message	Meaning	Fix suggestion
303	Missing UC sample – run cannot be analyzed.	In Plate setup in the ABI software none of the samples was named “UC”. Exactly one sample must be named “UC” for plate to be analyzed.	If you performed the PCR <i>with</i> a UC sample then open the real-time PCR file in the real-time PCR software, rename the UC sample wells “UC” and reload in the Bladder EpiCheck software. If you performed the PCR run <i>without</i> a UC sample repeat the PCR (see "Protocol for repeating PCR" page 77).
304	Too many NTC samples – run cannot be analyzed.	In Plate setup in the ABI software more than one of the samples was named “NTC”. Exactly one sample must be named “NTC” for plate to be analyzed.	Open the real-time PCR file in the real-time PCR software, rename the sample wells such that only one sample is named “NTC”, and reload in the Bladder EpiCheck software.
305	Too many UC samples – run cannot be analyzed.	In Plate setup in the ABI software more than one of the samples was named “UC”. Exactly one sample must be named “UC” for plate to be analyzed.	Open the real-time PCR file in the real-time PCR software, rename the sample wells such that only one sample is named “UC”, and reload in the Bladder EpiCheck software.
306	Invalid NTC sample – run cannot be analyzed.	In Plate setup in the ABI software one or more of the NTC sample wells was defined as omitted (prior to the PCR run). Patient samples can be analyzed only if the NTC sample is properly defined and is not contaminated.	Repeat the PCR (see "Protocol for repeating PCR" page 77) and make sure to include all the wells that participated in the PCR.

Table 34 (continued)

Error number	Error message	Meaning	Fix suggestion
307	Invalid NTC sample – run cannot be analyzed.	In Plate setup in the ABI software there is a mismatch between the detector names/dyes in the well. Table 1 defines the detector names and their corresponding dyes that are expected in each row. Patient samples can be analyzed only if the NTC sample is properly defined and is not contaminated.	One or more of the NTC wells has a detector mismatch. Follow the instructions in "How to fix detector mismatches" section, page 77 .
308	Invalid UC sample – run cannot be analyzed.	In Plate setup in the ABI software one or more of the UC sample wells was defined as omitted (prior to the PCR run). Patient samples can be analyzed only if the UC sample is properly defined and was successfully analyzed.	Repeat the PCR (see "Protocol for repeating PCR" page 77) and make sure to include all the wells that participated in the PCR.
309	Invalid UC sample – run cannot be analyzed.	In Plate setup in the ABI software there is a mismatch between the detector names/dyes in the well. Table 1 defines the detector names and their corresponding dyes that are expected in each row. Patient samples can be analyzed only if the UC sample is properly defined and was successfully analyzed.	One or more of the UC wells has a detector mismatch. Follow the instructions in "How to fix detector mismatches" section, page 77 .

Table 35 - Sample errors

Error number	Error message	Meaning	Fix suggestion
400	Inconsistent sample name – sample cannot be analyzed.	At least two of the sample wells have a different name. In order for the software to analyze a sample all its wells must have the same sample name.	Open the .sds file in the ABI software and in plate setup select the sample wells and rename such that all sample wells have the same sample name. <i>Note: an easy way to assign the same sample name to all wells of a column is to double-click the column number and set a sample name for all wells.</i>
401	Well has no data – sample cannot be analyzed.	One or more of the sample wells was omitted in plate setup in the ABI software prior to the PCR run. In order for the software to analyze a sample all its wells must be included in the plate setup.	Repeat PCR (see "Protocol for repeating PCR" page 77) and make sure all desired wells are included.
402	Incorrect detectors in well – sample cannot be analyzed.	There is a mismatch between the detector names/dyes in the well. Table 1 defines the detector names and their corresponding dyes that are expected in each row.	One or more wells of this sample has a detector mismatch. Follow the instructions in "How to fix detector mismatches" section, page 77 .

Table 35 (continued)

Error number	Error message	Meaning	Fix suggestion
403	Insufficient amount of DNA – sample cannot be analyzed.	The signal intensity of the IR marker in one or more of the sample wells was too low, reflecting there was insufficient DNA in those wells.	If this error occurred only in a single well due to forgetting to add DNA then repeat the PCR (see "Protocol for repeating PCR" page 77). If this error occurred in more than one well, collect a new urine sample from this patient and repeat the Bladder EpiCheck test.
405	Digestion control failed – sample cannot be analyzed.	Incomplete digestion of sample.	Collect a new urine sample from this patient and repeat the Bladder EpiCheck test.
406	DCR not in expected range – sample cannot be analyzed.	The signal intensity of the DCR marker is not within the expected range (too low).	Repeat PCR using a new Bladder EpiCheck Test Kit (see "Protocol for repeating PCR" page 77). In addition, please contact your local distributor and follow their instructions.
407	DCR not in expected range – sample cannot be analyzed.	The signal intensity of the DCR marker is not within the expected range (too high).	Repeat PCR using a new Bladder EpiCheck Test Kit (see "Protocol for repeating PCR" page 77). In addition, please contact your local distributor and follow their instructions.

Table 35 (continued)

Error number	Error message	Meaning	Fix suggestion
408	Inconsistent signal intensities of the IR marker – sample cannot be analyzed.	The 8 amplification curves of the IR marker in a certain sample are supposed to be very similar. A large difference between the signal intensities of IR in a certain sample might indicate the presence of PCR inhibitors in the sample.	Repeat PCR (see "Protocol for repeating PCR" page 77).
409	Inconsistent signal intensities of the IR marker – sample cannot be analyzed.	The 8 amplification curves of the IR marker in a certain sample are supposed to be very similar. A large difference between the signal intensities of IR in a certain sample might indicate the presence of PCR inhibitors in the sample.	There is an insufficient amount of DNA to repeat PCR, therefore collect a new urine sample from this patient and repeat the Bladder EpiCheck test.
415	Multiple samples with identical name - Sample cannot be analyzed	More than one column was the same sample name.	Open the real time PCR file in the real time PCR software, rename each sample such that each sample has a unique identification and reload it into the Bladder EpiCheck software.

12.2. Multiple errors

An EpiCheck run/sample/well might have multiple errors, each with its own fix suggestion. In case a certain sample gets two errors, one suggesting to repeat the PCR, and the other suggesting to collect a new urine sample from the patient, then collect a new urine sample from the patient.

12.3. Protocol for repeating PCR

This step is performed in cases of which after analysis of the EpiCheck run, the software instructs the user to repeat PCR due to one of the errors explained in section 12-Troubleshooting guide above.

The digested sample contains ~120µl. About 80µl of this sample is used in the Real-time PCR step. In order to repeat PCR it is required to have at least 88µl (considering pipetting errors).

Please perform the following:

1. Thaw the 1X Dilution buffer tube at room temperature (15-25°C). Once thawed mix the tube thoroughly by inverting a few times and briefly spin down to remove drops from inside the lid.
2. Add 1X Dilution buffer up to 88µl directly into digested DNA sample tube.
3. Prepare new UC and NTC samples by repeating step 10.3 DNA digestion ([page 37](#)). If another PCR batch is performed it is possible to combine the repeated samples to this PCR using the already prepared digested UC and NTC samples.
4. Repeat step 10.4 Real-time PCR ([page 39](#)).

Please note that potentially some samples might have insufficient DNA for analysis after dilution.

12.4. How to fix detector mismatches

If there was a detector mismatch:

1. Go to the ABI software, open the Bladder EpiCheck run template (under C:\Applied Biosystems\SDS Documents)
2. In the “Setup” tab choose all wells and copy them using Ctrl+C
3. Open your .sds file, go to the “Setup” tab, choose all wells and paste the information using Ctrl+V
4. Insert sample names again and save changes
5. Reload the .sds file in the Bladder EpiCheck software

13. References

1. Epigenetic modifications and human disease (2010), Portela A., Esteller M., Nat. Biotechnol. 28(10): 1057-68
2. Cancer epigenetics reaches mainstream oncology (2011), Rodriguez-Paredes M., Esteller M., Nat. Med. 17(3): 330-9
3. DNA methylation-based biomarkers in bladder cancer (2013), Kandimalla R, van Tilborg AA, Zwarthoff EC. Nat Rev Urol. 10(6): 327-35
4. EP17-A2: Evaluation of detection capability for clinical laboratory measurement procedures; approved guidelines – second edition (June 2016) Clinical and laboratory standards institutes (CLSI).
5. EP05-A3: Evaluation of precision of quantitative measurement procedures; approved guidelines –third edition (October 2014) Clinical and laboratory standards institutes (CLSI).
6. Witjes JA, Morote J, Cornel EB, Gakis G, van Valenberg FJP, Lozano F, Sternberg IA, Willemsen E, Hegemann ML, Paitan Y, Leibovitch I. Performance of the Bladder EpiCheck™ Methylation Test for Patients Under Surveillance for Non-muscle-invasive Bladder Cancer: Results of a Multicenter, Prospective, Blinded Clinical Trial. Eur Urol Oncol. 2018 Sep;1(4):307-313. doi: 10.1016/j.euo.2018.06.011. Epub 2018 Jul 17. PMID: 31100252.
7. Gallioli et al. DNA methylation urine biomarkers test (EpiCheck® assay) in the diagnosis of upper tract urothelial carcinoma: results from a single-center prospective study. J Urol 2021;206(3S) PD43-05.
8. Pierconti et al. Upper urothelial tract high-grade carcinoma: comparison of urine cytology and DNA methylation analysis in urinary samples. Hum Pathol. 2021 Dec;118:42-48. doi: 10.1016/j.humpath.2021.09.007

14. Glossary

Symbols

	Manufacturer		Harmful / irritant
	Catalogue number		Harmful / irritant
	Contains sufficient for <n> reactions		Health hazard
	Expire date DD-MM-YYYY		Corrosive
	Batch number		European conformity, in vitro diagnostic
	Control		
	Negative control		
	Positive control		
	Consult instructions for use		
	Temperature limitation		
	Upper temperature limitation		
	Authorized representative in the European community		

Bladder EpiCheck Software Symbols

987654929 Green underline - Negative analysis

039647415 Red underline - Positive analysis

987654929 Gray underline – Invalid

NTC NTC OK

NTC NTC failed/invalid

UC UC OK

UC UC failed/Invalid



Open patient report / Laboratory sample report



Well/Sample error (Mouse click for further information)



Selectable well



Menu



Analyze new file

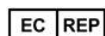
15. Contact information



The Bladder EpiCheck DNA Extraction Kit and the Bladder EpiCheck Test Kit are manufactured by: Nucleix Ltd. Pekeris 3, Rehovot 7670203, ISRAEL.

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For technical support please contact our technical support team at: support@nucleix.com