INSTRUCTIONS FOR USE

Article number/reference number CN 20201604

To achieve reliable results, these instructions must be followed precisely.

IVD

www.colonode.se/downloads/Instructions for Use

Article no. 60084-1.0

Content

1. Introduction

The ColoNode®-kit is for in vitro diagnostic (IVD) use.

1.1 Intended Use

The intended use is for detection of tumor cells in lymph nodes (LNs) of colorectal cancer (CRC) patients and determination of CRC tumor aggressiveness to aid when decisions are to be made about adjuvant chemotherapy and follow-up.

The ColoNode®-kit is a test intended for measurement of mRNA expression levels of five biomarker genes, i.e. CEACAM5 (carcinoembryonic antigen), KLK6 (kallikrein-related peptidase 6), SLC35D3 (solute carrier family 35 member D3), POSTN (periostin), and MUC2 (mucin 2) as well as amounts of 18S rRNA (18S ribosomal RNA) in total RNA extracted from LNs of CRC patients. The results are to be used as complementary information to the outcome of histopathology.

1.2. Background

Regional LN metastasis is the single most important prognostic risk factor for recurrence for patients who received curative surgery for CRC.[1-3] Biomarker mRNAs have great potential as tools for LN analysis in CRC. Analysis of biomarker expression at the mRNA level has several advantages over the protein level because several biomarkers can easily be analyzed in the same RNA extract and a large volume of the LN (potentially the entire LN) can be analyzed.[4]

In the ColoNode®-kit six target genes are analyzed. Four of these are expressed in CRC tumor cells, namely CEACAM5, KLK6, SLC35D3 and MUC2, and one, POSTN, is expressed in tumor associated fibroblasts. None of the genes are expressed in immune cells making them suitable for analysis of RNA extracted from LN samples. 18S rRNA is analyzed for normalization and as positive control.

Information about the target genes:
- CEACAM5 protein is a well-established tumor marker in adenocarcinoma and is used primarily for postoperative follow-up.[5] CEACAM5 is expressed in epithelial cells of the large bowel and their expression is retained in CRC.[5,6] CEACAM5 mRNA is expressed at high levels in tumor cells.[7,8] CEACAM5 mRNA was shown to be superior in detection of disseminated tumor cells in LNs of CRC patients compared to the routine method (microscopic examination of hematoxylin and eosin stained sections of LN tissue).[7-9] CEACAM5 mRNA levels are proportional to the number of disseminated tumor cells and high levels of CEACAM5 mRNA in LNs is an indicator of poor prognosis.[6-8] CEACAM5 mRNA can, when analyzed in combination with mRNA for the four biomarkers KLK6, SLC35D3, POSTN and MUC2, identify CRC patients at risk of recurrence with higher sensitivity than the routine method and allows allocation of CRC patients to different risk-groups.[10]
- KLK6 belongs to the human kallikrein-related gene family of serine proteases. Many of these are dysregulated in human malignancies and have a role in cell growth regulation, angiogenesis, invasion and metastasis.[11] KLK6 mRNA was identified as a promising progression biomarker for CRC. It is ectopically expressed in CRC tumor cells and detection of KLK6 mRNA in LNs is correlated to poor prognosis.[10,12]
- SLC35D3 is expressed in tumor cells but appears to have a different expression pattern from those of CEACAM5, MUC2 and KLK6.[10] Expression of SLC35D3 in LNs of CRC patients is associated with poor prognosis.[10]
- POSTN is expressed in fibroblasts and at elevated levels in stromal fibroblasts of CRC. The level of stromal POSTN in CRC primary tumor is a prognostic factor for CRC.[13] High levels of POSTN in LNs of CRC patients was associated with poor prognosis.[10]
- MUC2 is the major mucin in the mucous layer covering the colon and rectum epithelium. Ten to 20% of CRC tumors are mucinous, and have been shown to be associated with better prognosis than adenocarcinoma in general.[14] High MUC2 mRNA:CEACAM5 mRNA ratios in LNs of CRC patients was associated with good prognosis.[8,10]
- 18S rRNA is used for calculation of biomarker mRNA expression levels. 18S rRNA was proven to be expressed at relatively stable levels in lymphocytes and intestinal epithelial cells.[15,16]

The ColoNode®-kit was shown to detect all five biomarker mRNAs and 18S rRNA with equal efficiency in RNA extracted from both fresh-frozen- and formalin-fixed, paraffin-embedded LN tissue.[9]

1.3 Assay principle

The ColoNode®-kit is a dual 3-plex, one-step, real-time, quantitative reverse transcription-quantitative polymerase chain reaction (qRT-PCR) test for analysis of biomarker mRNA expression levels. It is designed and developed for the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems).
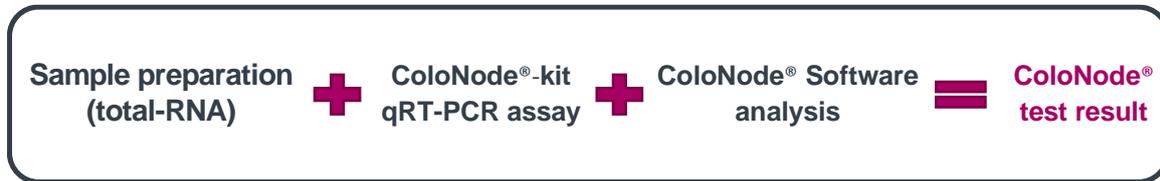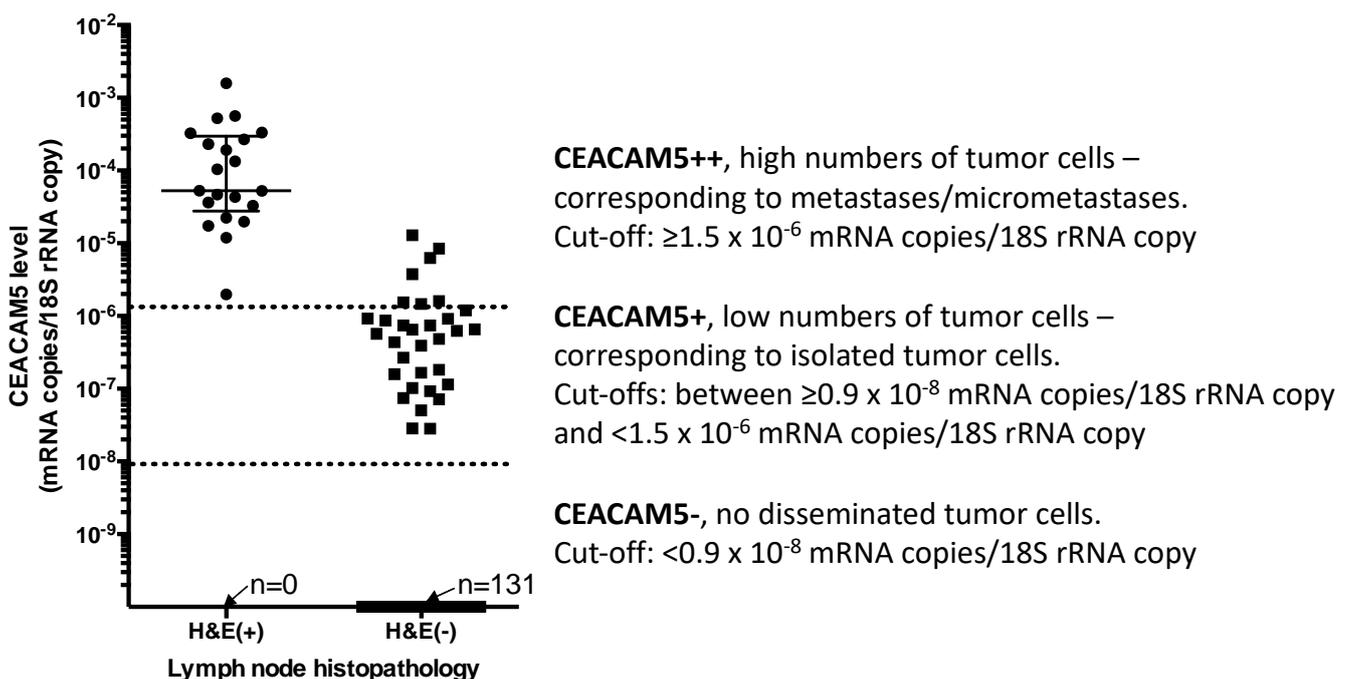

Figure 1: *Overview of ColoNode® procedure*

In the one-step qRT-PCR reaction both reverse transcription of RNA into cDNA and PCR amplification takes place in the same reaction mixture with all required reagents added initially, thereby avoiding contaminating agents and carry over effects. This is achieved by using the enzyme Tth DNA polymerase, which functions both as a reverse transcriptase and a DNA polymerase.

Starting material is total-RNA extracted from LN tissue. The five biomarker mRNAs and 18S rRNA are reverse transcribed to cDNA with specific 3'-primers that bind to the mRNA of the respective biomarker or the 18S rRNA and produces specific cDNAs. The amplification step utilizes a pair of 5'- and 3'-primers complementary to one strand each and a biomarker mRNA specific probe that targets one of the strands in the cDNA located between the primers. The probe is labeled with a fluorescent reporter dye at the 5'-end and a quencher dye at the 3'-end that prevents fluorescence emission from the intact probe. During amplification the Tth DNA polymerase will cleave cDNA-bound probe thereby releasing the fluorescent reporter dye, increasing the fluorescence intensity with each PCR-cycle. This process is monitored in real-time. When the intensity of the fluorescence signal reaches a pre-defined threshold, the number of PCR cycles required to reach the threshold is referred to as the cycle threshold (ct).

The ColoNode®-kit raw data result from the qRT-PCR run is transferred from the QuantStudio™ 5 Real-Time PCR System and analyzed by the ColoNode® Software yielding tumor cell detection calculated as described in reference 8 and estimation of CRC tumor aggressiveness calculated as described in reference 10, i.e. the formula given therein is applied on the LN with the highest CEACAM5 mRNA level (copies/18S rRNA copy). In addition, for every sample the mean concentration in copies/µL is given for the five biomarker mRNAs and for 18S rRNA.

CEACAM5++, high numbers of tumor cells – corresponding to metastases/micrometastases.
Cut-off: ≥1.5 x 10^{-6} mRNA copies/18S rRNA copy

CEACAM5+, low numbers of tumor cells – corresponding to isolated tumor cells.
Cut-offs: between ≥0.9 x 10^{-8} mRNA copies/18S rRNA copy and <1.5 x 10^{-6} mRNA copies/18S rRNA copy

CEACAM5-, no disseminated tumor cells.
Cut-off: <0.9 x 10^{-8} mRNA copies/18S rRNA copy

Figure 2: *Cut-offs for CEACAM5 levels. Each dot represents a lymph node analyzed side-by-side by routine histopathology (H&E) and CEACAM5 mRNA level by ColoNode®. Data from reference 9.*

1.4 Assay design

Each kit is designed for analysis of the LNs of one patient only. The ColoNode®-kit performs the reverse transcription, the amplification and the quantitation of mRNA by qRT-PCR with two different 3-plex reaction mixes run in parallel. The kit contains two master mixes that include primers and probes for three of the biomarkers in each. It also contains standard samples (STDs) with mixes of three of the biomarkers in each at defined RNA copy concentrations yielding standard curves for all six biomarkers, allowing determination of the concentration in RNA copies/µL. Finally, it contains no template controls (NTCs) for the two 3-plex assays. NTCs, STDs and unknown LN samples are all analyzed in duplicate.

Specific 5'- and 3'-primers have been constructed for each of the 5 biomarker mRNAs and for 18S rRNA. They are placed in different exons of the respective biomarker gene. A sequence-specific dye-labeled probe is placed over the boundary between the two exons to make the assay RNA specific, thereby avoiding amplification of genomic DNA. Three different fluorescence dyes (FAM™, VIC™, NED™) are used to perform quantitation of 3 biomarkers in a single reaction. Three of the biomarkers are measured in the left half of the plate and 2 of the biomarkers and 18S rRNA are measured in the right half. Carboxy-X-rhodamine (ROX) is used as a reference dye for normalization of the fluorescent reporter signals in the qRT-PCR run.

STDs containing specified amounts of RNA copies from each of the biomarkers are analyzed in parallel to unknown LN samples to generate standard curves. The analyzed unknown samples get the actual copy number/µL calculated from the respective standard curve. The RNA copy standards also serve as positive controls of the ColoNode®-kit performance. NTCs for each master mix serve as controls for contamination. qRT-PCR analysis of 18S rRNA in unknown samples serve as a quality control of the analyzed RNA and for calculation of the mRNA levels.

NOTE! *Samples with 18S rRNA concentrations below 10^8 copies/µL should be interpreted with caution due to risk for false negative results. ColoNode® Software will alarm by marking samples with an 18S rRNA-value less than 10^8 copies/µL as "Invalid".*

2. Materials and Equipment

2.1 Content

The ColoNode®-kit contains ready for use reagents for analysis with the two 3-plex qRT-PCR systems of 20 unknown samples of total-RNA extracts of LNs, 6 RNA copy standard samples (STDs) and 2 no template controls (NTCs).

Table 1. *Content of the ColoNode®-kit*

Reagent	Tube labelling	No. of tubes	Cap color	Volume (µL/tube)	Storage condition
Mastermix for system 1	ColoNode® Mastermix 1	1	Green	790	-18°C/-25°C
Mastermix for system 2	ColoNode® Mastermix 2	1	Blue	790	-18°C/-25°C
Standards (STDs) for system 1	ColoNode® Standard 1 Tube A, Tube B, Tube C	3	Green	5	-18°C/-25°C
Standards (STDs) for system 2	ColoNode® Standard 2 Tube D, Tube E, Tube F	3	Blue	5	-18°C/-25°C
Manganese acetate (25 mM)	Manganese Solution	1	White	165	-18°C/-25°C
No template control (NTC)	Negative control	1	Grey	20	-18°C/-25°C

NOTE! *A maximum of 20 LN samples can be analyzed by the kit. Use additional kits if more than 20 LNs need to be analyzed and combine the results. For each ColoNode®-kit qRT-PCR run the results are presented as detected tumor cells by the CEACAM5 mRNA level given for each LN and can directly be combined. One tumor aggressiveness factor will be given for each ColoNode®-kit run. The highest tumor aggressiveness factor should then represent the result for the patient. The kit should only be used for LNs from one patient.*

2.2 Materials required, but not provided

General laboratory equipment

The following general laboratory equipment is required to perform ColoNode®-kit analysis:

- Vortex
- Pipettes (range 0.5-10 µL, 10-100 µL and 100-1000 µL)

- Bench top centrifuge with rotor for 1.5-2 mL tubes
- A centrifuge with rotor for 96-well PCR-plates
- An RNase-free working area
- Ice-bath for thawing and handling of tubes prior to analysis

All laboratory equipment should be installed, calibrated, operated and maintained according to the manufacturer's instructions and recommendations.

General laboratory consumables and reagents
The following general laboratory consumables are required to perform ColoNode®-kit analysis:
- 1.5 mL tubes for PCR-use
- Pipette tips with aerosol barrier for PCR-use
- Sterile powder-free disposable gloves

Recommended special equipment and consumables
- QuantStudio™ 5 Real-Time PCR System with block for 0.2 mL, 96-well plates (Standard block, catalog #A28139, Applied Biosystems). Installation, calibration and maintenance shall be performed according to manufacturer's instructions and recommendations.
- Equipment for measurement of RNA concentration and quality.
- 96-well plate and plastic cover (MicroAmp™ Optical 96-Well Reaction Plate with Barcode & Optical Adhesive Films catalog #4314320 for Quant Studio™ 5, Applied Biosystems).
- Applicator to seal the plastic cover on the 96-well plate (MicroAmp™ Adhesive Film Applicator catalog #4333183, Applied Biosystems).
- RNase-free water, optional with RNase inhibitor, when dilution of samples is required.

2.3 Softwares
- QuantStudio™ 5 Design and Analysis Software for qRT-PCR analysis.
- ColoNode® Software for analysis of ColoNode® kit qRT-PCR raw data from the Quant Studio software. Find ColoNode® Software at www.colonode.se and use the login information supplied on the delivery note.

2.4 Downloads
Information available for download from www.colonode.se are:
- ColoNode®-kit Instructions for use (IFU)
- Set-up template for ColoNode®-kit qRT-PCR runs on QuantStudio™ 5
- ColoNode® chemical information and safety data sheet

3. Storage requirements

- The ColoNode®-kit reagents are stable until the expiration date when stored and handled as described in this IFU.
- Do not use the kit after expiration date.
- Do not mix kit components from the same or different lot numbers.
- The kit is intended for one time use only.
- Avoid freezing-thawing.
- Store the ColoNode®-kit at -18°C/-25°C

4. Precautions and safety

- The ColoNode®-kit procedure should be performed according to this IFU.
- Deviations from this IFU can compromise the kit performance and results.
- It is important to follow the instructions when exporting the results from QuantStudio™ 5, otherwise the ColoNode® Software results can be compromised.
- <u>Do not</u> pool reagents with different kit lot numbers or different vials of the same lot.
- <u>Do not</u> use damaged reagent vials.

- The ColoNode®-kit contains no ingredients at hazardous concentrations. For more information please read the ColoNode® chemical information and safety data sheet available at our website (www.colonode.se).
- Use of this product should be limited to personnel with qualifications in handling of RNA and real-time PCR analysis.
- When working with the ColoNode®-kit always wear a laboratory coat and powder free disposable gloves.
- Frozen components should be completely thawed on ice.
- All reagents shall be kept on ice during the kit procedure.
- Disposal of kit components should be in accordance with the national biohazard safety guidelines, country, federal, state and local regulations.
- Avoid microbial contamination and prevent introduction of RNases into kit components and samples.
- It is highly recommended to keep strict separation when preparing ColoNode® master mix, RNA samples and qRT-PCR runs in the laboratory in order to avoid cross-contamination. Furthermore, all laboratory equipment should be dedicated to each area and not be moved between the different working areas.
- Dispose all PCR amplified 96-well plates immediately after the qRT-PCR run without opening the plastic cover in order to avoid cross-contamination.
- Keep working areas clean and free from RNases.
- Use sterile pipette tips with aerosol barriers and change tip between every sample to prevent cross-contamination between samples.
- The ColoNode®-kit does not contain any infectious substances or agents, which can cause disease in humans or animals.

5. Procedural limitations

The ColoNode®-kit:
- is developed for analysis of total-RNA extracts of LN tissue of CRC patients
- results might be affected if the input RNA quality and concentration deviates from the recommendations in the IFU
- will only measure the six described biomarkers
- a maximum of 20 LN samples can be analyzed per kit
- one kit should be dedicated to the LNs from one patient since tumor aggressiveness is calculated from the biomarker expression profile of the LN with the highest CEACAM5 mRNA level

6. Sample requirements

The ColoNode®-kit is intended for analysis of total-RNA extracts. The purity and concentration of sample input RNA is crucial for successful use of the ColoNode®-kit. It is advisable to save the RNA extract until you have seen the results of the analysis in order to allow for a possible re-run.

6.1 Samples
The performance of the ColoNode®-kit has been validated on total-RNA extracted from fresh-frozen half LNs of CRC patients and from 80μm sections of formalin-fixed, paraffin-embedded LNs of CRC patients (reference 9).

6.2 Homogenization and RNA extraction
For large LN tissue volumes like half LNs, fresh-frozen or formalin fixed, the first step is to homogenize the LN tissue by using a method that provides a homogeneous solution of the entire LN tissue sample. In the second step, the homogeneous solution, or an aliquot thereof, is used for extraction of total-RNA. The homogenization step is not required for extraction of RNA from smaller LN tissue volumes, e.g. sections of formalin-fixed, paraffin-embedded LNs. Total-RNA extraction should be performed according to the instructions for use of the manufacturer, whose kit is used. Recommendations for extraction of RNA:
- For fresh-frozen LNs: extract total-RNA by using the acid-guanidine-phenol-chloroform method.[17]
- For formalin-fixed LNs: the Roche Highpure FFPET RNA isolation kit (Catalog no. 06650775001, Roche) can be used for total-RNA extraction from both large tissue volumes as half-LNs and LN tissue sections. For thin sections, the RNeasy DSP FFPE Kit (Catalog no: 73604, Qiagen) can also be used.

6.3 RNA purity and concentration

Total-RNA concentration should be determined by measuring optical density at 260 nm (OD260) and purity estimated as OD260/OD280 ratio, e.g. by using a NanoDrop instrument. The concentration of total-RNA in samples should be within the interval of 10 – 1200 ng/µL. Dilute the total-RNA sample to be within interval. Concentrations between 500 – 1000 ng/µL, are preferred. *NOTE! Dilution is only necessary if concentration is > 1200 ng/µL.* Use RNase-free water for dilution, optional with RNase inhibitor. RNA samples should be free from potential PCR inhibiting factors. OD260/OD280 ratio should be >1.8.

7. Instructions for use

ColoNode®-kit procedure
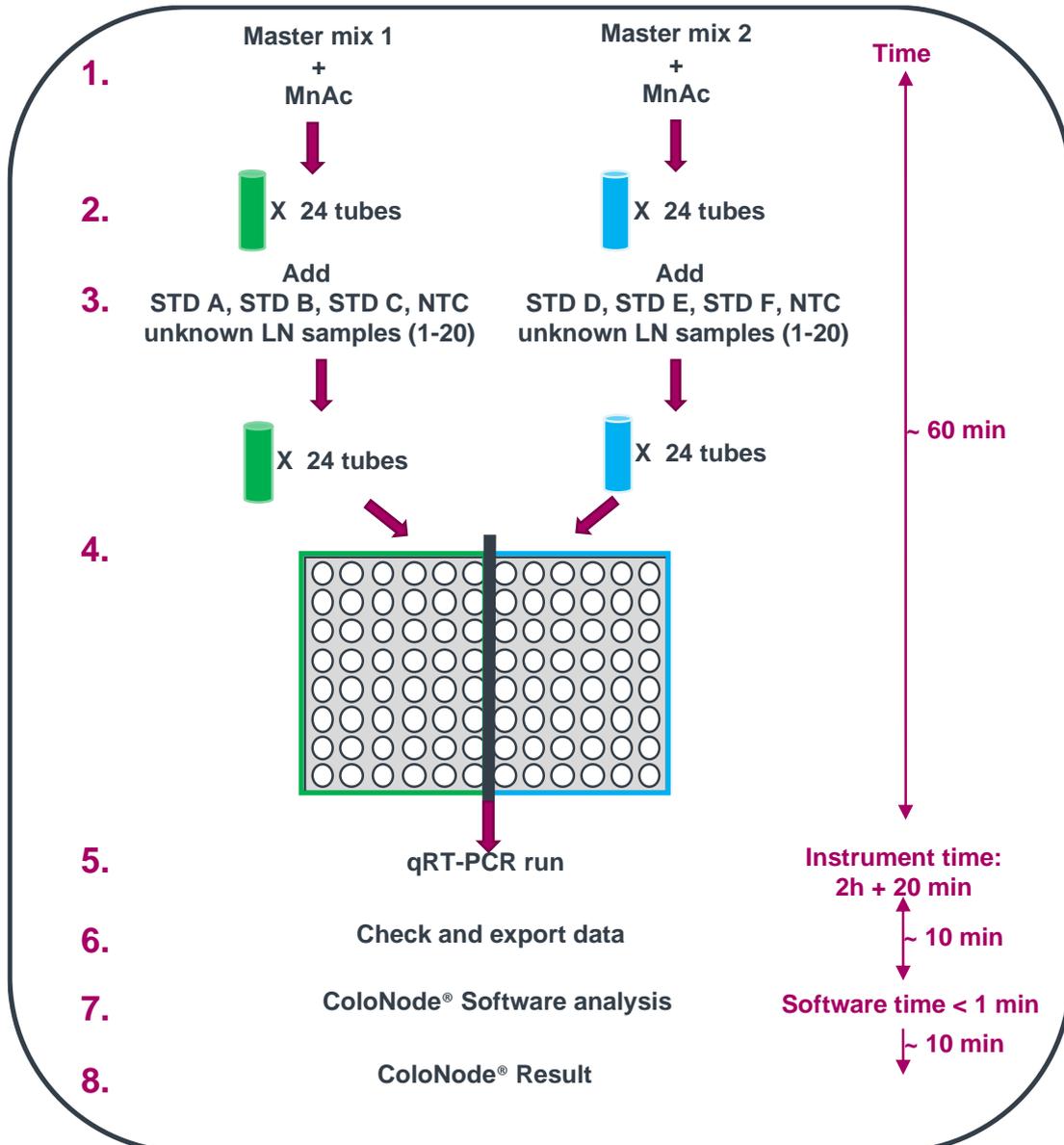
Figure 3. *1) Add MnAc-solution to the master mix tubes for system 1 and 2; 2) Aliquot each of the two MnAc containing master mixes to 24 tubes labelled according to Figure 4; 3) Add RNA to respective tube, i.e. 3 standard (STD) samples unique for system 1 and system 2 (tubes A, B, C and D, E, F, respectively), 1 negative control (NTC) and 20 unknown LN samples per master mix, 4) Distribute from the tubes to a 96-well plate placing the samples at the positions shown in Figure 5; 5) Run the plate in the qRT-PCR instrument; 6) Inspect the qRT-PCR run and export the data from the instrument computer; 7) Use the exported file as input for the ColoNode® Software and analyze data, and 8) Receive ColoNode® results.*

7.1 ColoNode®-kit procedural guidelines

- Perform all steps on ice.
- Use disposable, sterile plastic ware and sterile, disposable RNase-free tips and tubes.

- Wear disposable powder-free gloves while handling reagents and RNA samples in order to prevent RNase contamination from the skin. Change gloves frequently.
- Always use proper microbiological aseptic techniques when working with RNA.
- Work in an RNase-free area.

7.2 ColoNode®-kit procedure

1. Download the set-up template for the qRT-PCR run from www.colonode.se to a USB stick or directly to the PC-computer coupled to the QuantStudio™ 5 Real-Time PCR System. instrument
2. The set-up template is locked. Before you open the template file you need to make a copy and give it a patient unique code name.
3. Open the copy with the patient unique code name and click OK to continue.
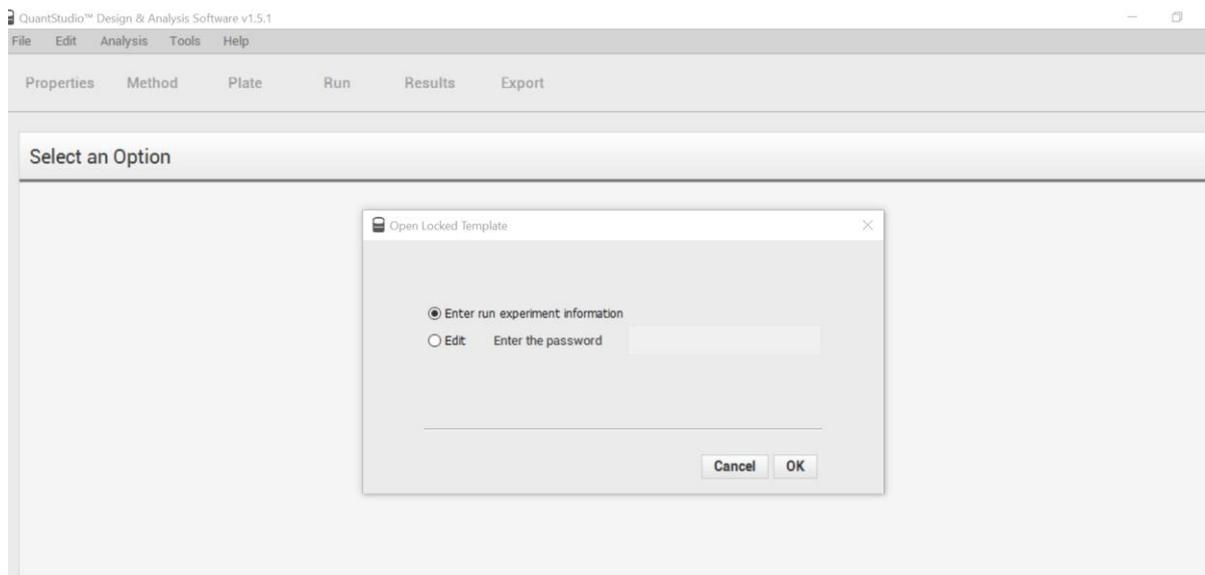
4. Under the tab "**PROPERTIES"** in the window **"Experiment properties"** enter the same patient unique code under "**Name"** and optionally enter "**User name**" and save the file.
5. Prepare 24 RNase-free, 1.5 mL tubes for each of the two master mixes for system 1 and 2, respectively (green and blue coded). Label them as described in Figure 4: one tube per RNA standard concentration (tube STD A, B, C or STD D, E, F, respectively), two tubes per unknown RNA sample to be analyzed, one for each master mix (M1/S1 to M1/S20 and M2/S1 to M2/S20, respectively) and NTC-M1 and NTC-M2. Thus, for a full plate you should totally have 48 tubes, 24 tubes/master mix (Figure 4).

Labelling of tubes
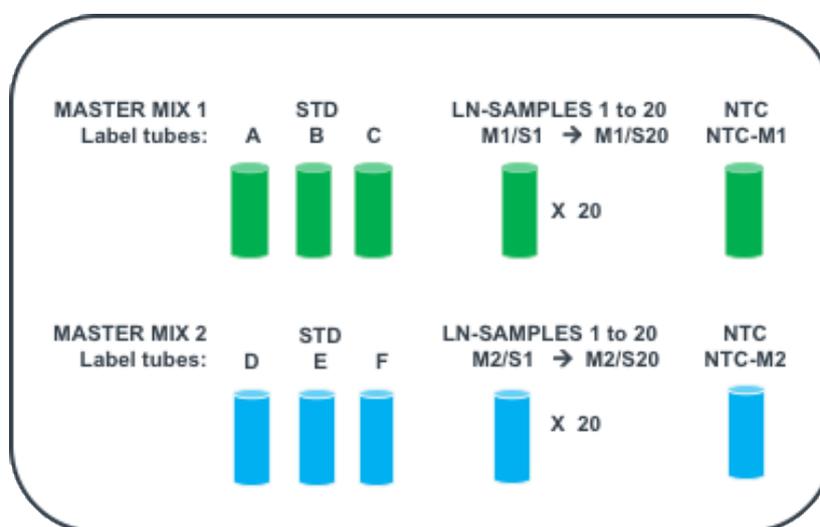
Figure 4. For <u>each</u> master mix: Label tubes for 1 NTC (NTC-M1, NTC-M2), 3 STD concentrations (A, B, C and D, E, F, respectively) and 20 unknown LN samples (M1/S1 to M1/S20 and M2/S1 to M2/S20, respectively), i.e. totally 24 tubes/master mix and 48 tubes for the entire qRT-PCR run.

6. Thaw all reagent tubes and keep them on ice (Master mix 1 and Master mix 2, Manganese-solution, NTC and STDs for system 1 and 2). Thaw your samples and keep them on ice.
7. When thawed, invert all tubes a couple of times. Centrifuge briefly in a benchtop centrifuge prior to use to get the entire volume of reagent at the bottom of the tube.
8. Activate the master mixes for system 1 and system 2 by adding 124 µL Manganese-solution per master mix tube. Vortex the tubes shortly (~5 sec.) and centrifuge down the solution.
9. Aliquot 37 µL of activated master mix solution to the 24 tubes labelled for system 1 (green) and system 2 (blue), respectively. Put the tubes on ice.
10. Add 3 µL of STD or 3µL of unknown RNA sample or 3 µL of NTC to the labelled tubes with activated Master mix 1 and Master mix 2. Vortex shortly (~5 sec.) and centrifuge down the solution.
11. Distribute the volume from each tube with activated master mix containing RNA by pipetting 19 µL/well into 2 wells in the 96-well qRT-PCR plate according to the scheme depicted in Figure 5. Ensure that the samples are entered in the correct wells. If you have fewer than 20 LN samples, leave the residual wells dedicated for samples empty.
    The negative controls (NTCs) should always be at the positions shown in Figure 5, i.e. NTC-M1 in wells H5–H6 and NTC-M2 in wells H11 –H12.
    If you have more than 20 LN samples, see NOTE in section 2.1, page 5 for instructions.

SAMPLE POSITIONS IN THE 96-WELL qRT-PCR PLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 Tube A	STD 1 Tube A	STD 1 Tube B	STD 1 Tube B	STD 1 Tube C	STD 1 Tube C	STD 2 Tube D	STD 2 Tube D	STD 2 Tube E	STD 2 Tube E	STD 2 Tube F	STD 2 Tube F
B	M1/S1	M1/S1	M1/S2	M1/S2	M1/S3	M1/S3	M2/S1	M2/S1	M2/S2	M2/S2	M2/S3	M2/S3
C	M1/S4	M1/S4	M1/S5	M1/S5	M1/S6	M1/S6	M2/S4	M2/S4	M2/S5	M2/S5	M2/S6	M2/S6
D	M1/S7	M1/S7	M1/S8	M1/S8	M1/S9	M1/S9	M2/S7	M2/S7	M2/S8	M2/S8	M2/S9	M2/S9
E	M1/S10	M1/S10	M1/S11	M1/S11	M1/S12	M1/S12	M2/S10	M2/S10	M2/S11	M2/S11	M2/S12	M2/S12
F	M1/S13	M1/S13	M1/S14	M1/S14	M1/S15	M1/S15	M2/S13	M2/S13	M2/S14	M2/S14	M2/S15	M2/S15
G	M1/S16	M1/S16	M1/S17	M1/S17	M1/S18	M1/S18	M2/S16	M2/S16	M2/S17	M2/S17	M2/S18	M2/S18
H	M1/S19	M1/S19	M1/S20	M1/S20	NTC-M1	NTC-M1	M2/S19	M2/S19	M2/S20	M2/S20	NTC-M2	NTC-M2

Green: System 1, i.e. samples in Master mix 1 (M1)   **Blue: System 2, i.e. samples in Master mix 2 (M2)**

Figure 5. Sample positions in the 96-well qRT-PCR plate. At the left side (green) the samples are diluted in master mix 1 (M1) and analyzed for the biomarkers of System 1, at the right side (blue) the samples are diluted in master mix 2 (M2) and analyzed for the two biomarkers of System 2 and 18S rRNA. STD1 = RNA copy standard for System 1; STD 2 = RNA copy standard for System 2; NOTE! Each LN total-RNA extract sample is analyzed in duplicate for System 1 (M1/S1 to M1/S20) and System 2 (M1/S1 to M2/S20), respectively; NTC-M1 = no template control for system 1; NTC-M2 = no template control for system 2.

12. Seal the plate with a plastic cover according to QuantStudio™ 5 manual.
13. Centrifuge the 96-well plate briefly in a benchtop centrifuge for 15 to 30 seconds.
14. Switch on the power of the QuantStudio™ 5 instrument. (It is important that the computer is switched on before the qRT-PCR instrument).
15. Put the 96-well plate in the instrument.

16. Go to your setup file (.edt). On the upper tab, press "**METHOD"** to check that the following PCR-profile is prepared:
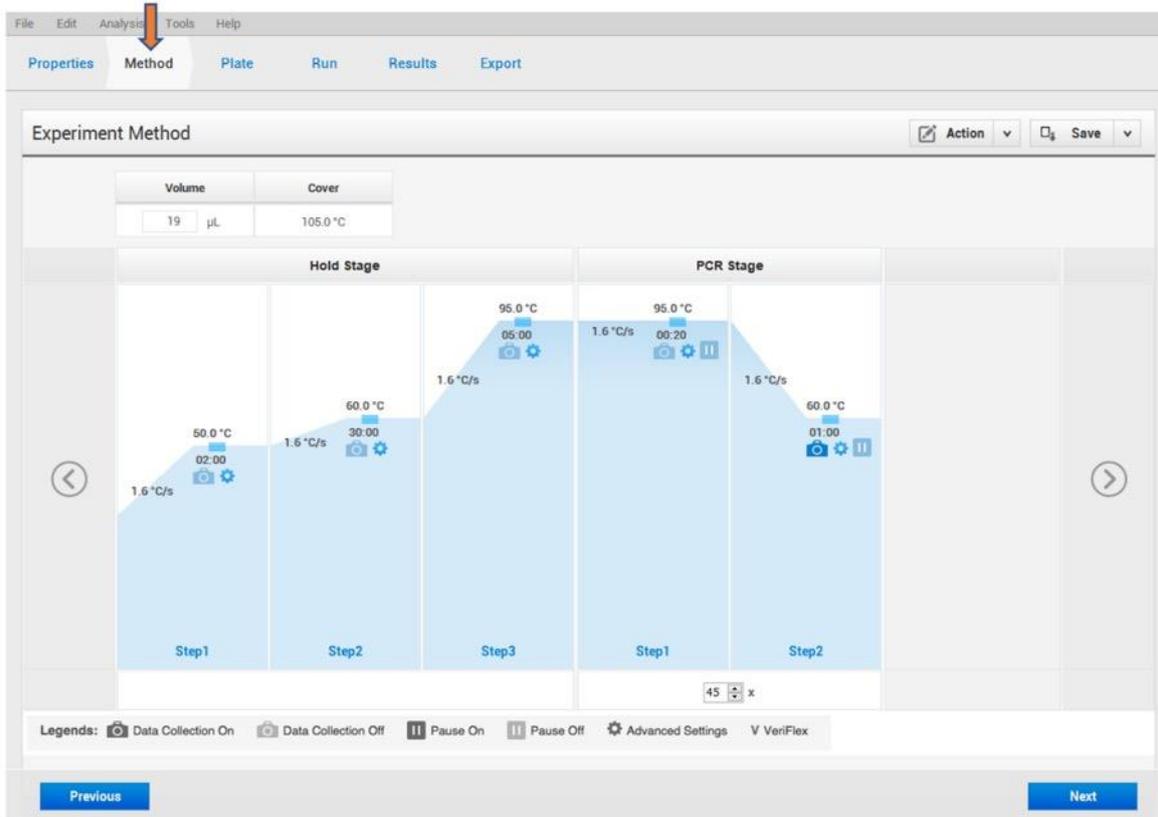

17. On the upper tab, press "**RUN**". Start the run by clicking on the blue button named "**START RUN**", chose the serial number of QS5. Save the run with a patient unique code (.eds) by pressing "**Save**" in the box that appears.
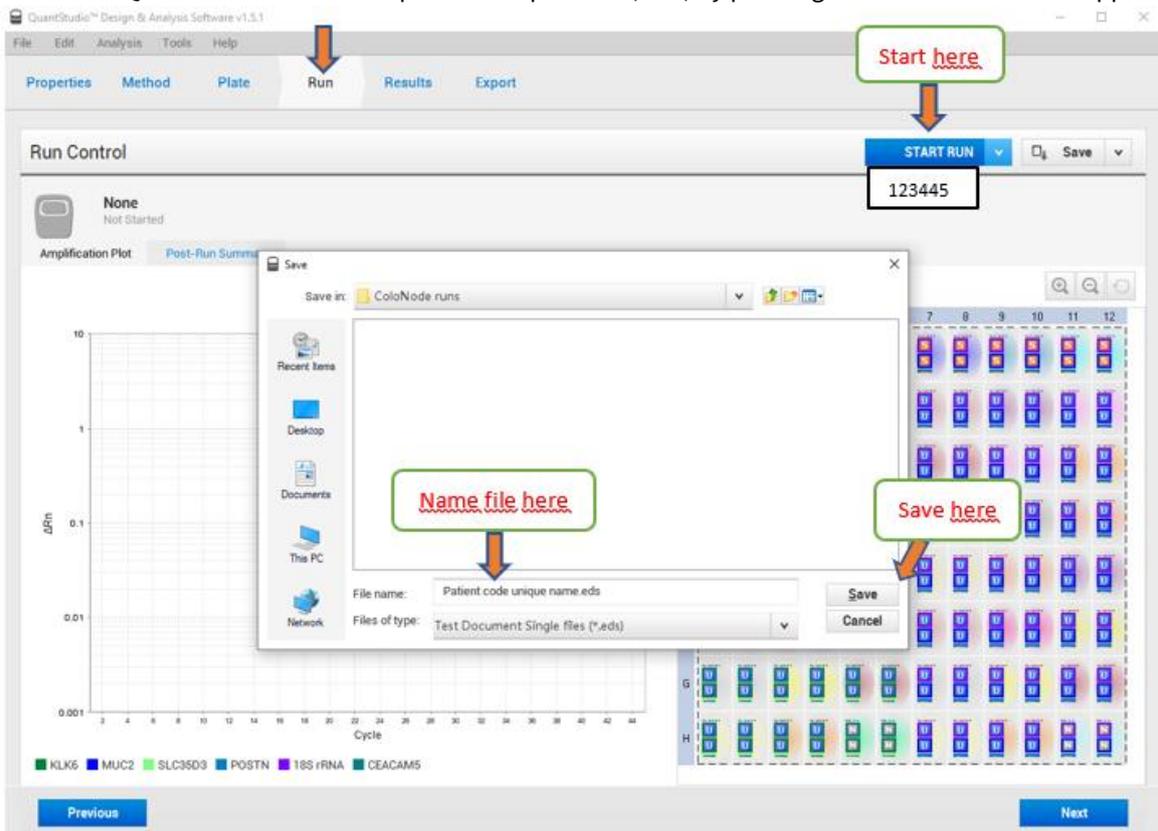
18. The run takes approximately 2 hours and 20 minutes.

7.3 Instrument result analysis

1. When the qRT-PCR run is finished, start by saving the raw data results.
2. *Export Excel-file (.xls) by*:
   - On the upper tab, press "**EXPORT".**
   - **Check that the filename is there otherwise fill it in**.
   - Under "**CONTENT"**, mark "**RESULTS"**
   - Browse to a location for the excel-file on the computer next to "**LOCATION"**
   - Press **"EXPORT"**
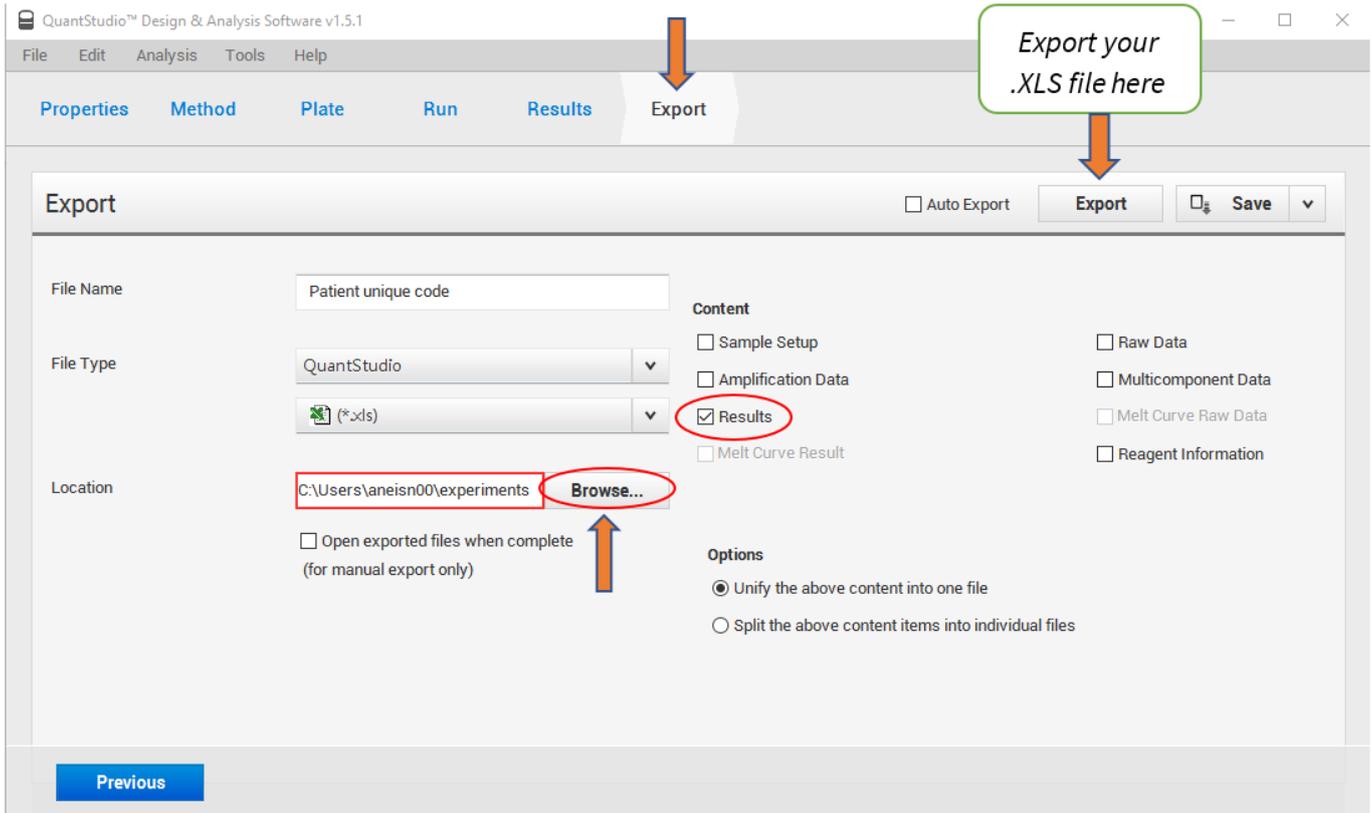
3. Make a copy of the raw data file, for eventual troubleshooting in the future.
4. Transfer the exported Excel-file (.xls) to a computer that is connected to internet in order to access the ColoNode® Software at the ColoNode homepage.

7.4 ColoNode® Software procedure

Analysis of results using the ColoNode® software:

1. Log in to ColoNode® Software by using the details received on the delivery note.
2. Upload the exported Excel-file (.xls) from step 4 under 7.3 by pressing "**Select file**"(black button), enter the lot-number and press "**Upload file**" (blue button).
3. Press "**Analyze file**" (turquoise button). When the calculations are ready there will be a green sign saying: "**OK Analysis successful**". Scroll down to see the COLONODE TEST RESULT SUMMARY containing:
   -"Tumor cell detection in lymph nodes and tumor aggressiveness", summary
   -"Tumor cell detection", CEACAM5 level as estimate of tumor cell load in the individual lymph nodes
   -Concentration of biomarker mRNAs and 18S rRNA in copies/µL in the individual lymph node RNA extracts
   -"Quality control of standard curve slopes and No Template Controls", summary
   Details can be viewed by pressing the "**Information**"-buttons.
4. To save the result press the black box with a "**Download**"-symbol to the right of the file name. All results will then be saved and downloaded in a pdf-file. The pdf-file contains the COLONODE TEST RESULT SUMMARY, the Tumor Cell Detection as CEACAM5 mRNA level for each lymph node sample, the concentration of the five biomarker mRNAs and

18S rRNA in each lymph node, summary of control of standard curve slopes and no template control for all six biomarkers and a list of ct- and quantity-values for each qRT-PCR run of unknown samples and standard curve points as well as the result of quality controls. The concentration of the five biomarker mRNAs and 18S rRNA in each lymph node can also be saved and downloaded as an Excel-file by pressing the green box with an Excel-symbol.

THE FOLLOWING ASPECTS WILL BE CHECKED BY THE COLONODE SOFTWARE

No signal in NTCs ✓   Standard curves OK ✓

Sample duplicates OK ✓   Enough 18S rRNA in samples ✓
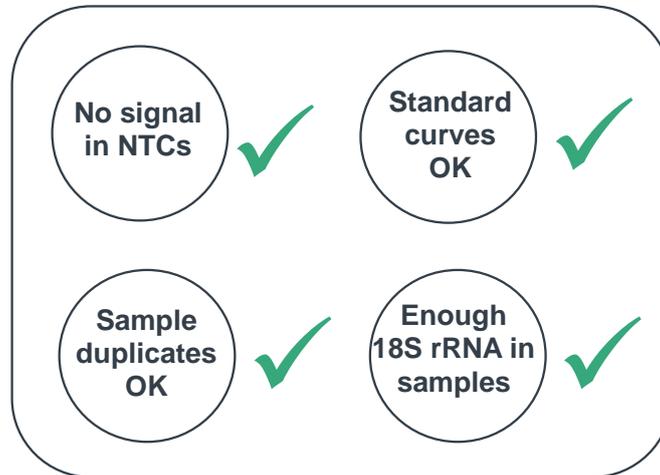
How to interpret the quality controls:

✓ Result OK. No further analysis needs to be done.

! Press the "**Information"-**button. Read the reason(s) for the warning and evaluate if a re-run is needed or if the results still can be used.

8. Meaning of symbols used on labels

IVD In vitro diagnostics

LOT Lot or batch number

REF Catalogue number

Expiry date

Consult IFU supplied at the homepage

e-instructions

Manufacturer

Storage temperature

CE Conformitè Europëenne mark

9. Notice to purchaser

This product was manufactured using NED™ Phosphoamidites and/or VIC™ Phosphoramidites. Both NED™ and VIC™ are trademarks of Thermo Fisher Scientific Inc. MGB™, MGB Eclipse® and Eclipse® are trademarks of EliTechGroup® Inc.

10. References

1. Chang GJ, Rodriguez-Bigas MA, Skibber JM, Moyer VA. Lymph node evaluation and survival after curative resection of colon cancer: systematic review. J Natl Cancer Inst. 2007;99:433-441.
2. Iddings D, Bilchik A. The biologic significance of micrometastatic disease and sentinel lymph node technology on colorectal cancer. J Surg Oncol. 2007;96:671-677.
3. Nicastri DG, Doucette JT, Godfrey TE, Hughes SJ. Is occult lymph node disease in colorectal cancer patients clinically significant? A review of the relevant literature. J Mol Diagn. 2007;9:563-571.
4. Hammarström S. Biomarker mRNAs as prognostic tools for lymph node analysis in colorectal cancer. Biomark Med. 2019;13:801-803.
5. Hammarström S. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. Semin Cancer Biol. 1999;9: 67-81.
6. Ohlsson L, Hammarström M-L, Israelsson A, Näslund L, Öberg Å, Lindmark G, Hammarström S. Biomarker selection for detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR. Br J Cancer. 2006;95:218-225.
7. Öberg Å, Lindmark G, Israelsson A, Hammarström S, Hammarström M-L. Detection of occult tumor cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR for CEA and CK20 mRNAs. Int J Cancer. 2004;111:101-110.
8. Ohlsson L, Israelsson A, Öberg Å, Palmqvist R, Stenlund H, Hammarström M-L, Hammarström S, Lindmark G. Lymph node CEA and MUC2 mRNA as useful predictors of outcome in colorectal cancer. Int J Cancer. 2012;130:1833-1843.
9. Olsson LM, Lindmark GE, Israelsson ACE, Korkocic D, Hammarström SG, Hammarström M-LKC. CEACAM5, KLK6, SLC35D3, POSTN and MUC2 mRNA analysis improves detection and allows characterization of tumor cells in lymph nodes of colon cancer patients. Dis Colon Rectum. 2021;64:1354-1363. doi: 10.1097/DCR.0000000000002151.
10. Olsson L, Hammarström M-L, Israelsson A, Lindmark G, Hammarström S. Allocating colorectal cancer patients to different risk categories by using a five-biomarker mRNA combination in lymph node analysis. PLoS ONE. 2020;15:e0229007.
11. Borgono C, Diamandis E. The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer. 2004;4:876-890.
12. Ohlsson L, Lindmark G, Israelsson A, Palmqvist R, Öberg Å, Hammarström M-L, Hammarström S. Lymph node tissue kallikrein-related peptidase 6 mRNA – a progression marker for colorectal cancer. Br J Cancer. 2012;107:150-157.
13. Oh HJ, Bae JM, Wen X-Y, Cho N-Y, Kim J-H, Kang GH. Overexpression of POSTN in tumor stroma is a poor prognostic indicator of colorectal cancer. J Pathol Transl Med. 2017;51:306-313.
14. Liu Q, Luo D, Li Q, Zhu J, Li X. Evaluating the effect of lymph node status on survival in large colon cancer. Front Oncol. 2018;8:602.
15. Bas A, Forsberg G, Hammarström S, Hammarström M-L. Utility of the housekeeping genes 18S rRNA, β-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. Scand J Immunol. 2004;59:566–573.
16. Fahlgren A, Hammarström S, Danielsson Å, Hammarström M-L. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. Clin Exp Immunol. 2003;31:90-101.
17. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenolchloroform extraction. Anal Biochem, 1987;162:156-159.

11. Abbreviations

CEACAM5, carcinoembryonic antigen, carcinoembryonic antigen-related cell adhesion molecule 5
CRC, colorectal cancer
Ct, cycle threshold
IFU, instructions for use
KLK6, kallikrein-related peptidase 6
LN, lymph node
MUC2, mucin 2
NTC, no template control
OD, optical density
POSTN, periostin
qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction
ROX, carboxy-X-rhodamine
SLC35D3, solute carrier family 35 member D3
STD, standard

12. Contact information

The ColoNode®-kit and software are manufactured by:
HiloProbe AB, Tvistevägen 48C, Umeå, SE- 90736, Sweden

For further information and support please contact your local distributor or contact HiloProbe by sending an email to info@colonode.se or by calling the phone number +46768216770.

The company HiloProbe AB holds a patent for the ColoNode®-kit.