

INSTRUCTIONS FOR USE



Article number/reference number CN 20201604

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



www.colonode.se/product

HiloProbe AB

www.hiloprobe.se; www.colonode.se
Tvistevägen 48C, SE-90736 Umeå, Sweden
COPYRIGHT© HiloProbe AB, 2024
Version 6, 2024-03-01

Content	Page
1. Introduction	3
1.1 Intended Use	3
1.2 Background	3
1.3 Assay principle	4
1.4 Assay design	5
2. Materials and Equipment	5
2.1 Content.....	5
2.2 Materials required, but not provided	6
2.3 Softwares.....	6
2.4 Downloads	6
3. Storage requirements	6
4. Precautions and safety	7
5. Procedural limitations	7
6. Sample requirements	7
6.1 Samples	7
6.2 Homogenization and RNA extraction	8
6.3 RNA purity and concentration	8
7. Instructions for use	8
7.1 ColoNode® kit procedural guidelines	9
7.2 ColoNode® procedure	9
7.3 Export of qRT-PCR instrument result	13
7.4 ColoNode® Software procedure	13
7.5 ColoNode® Software quality controls	14
7.6 ColoNode® result evaluation	14
7.7 Interpretation of multiple ColoNode® analyses for the same patient.....	14
8. Meaning of symbols used on labels	15
9. Abbreviations	15
10. Notice to purchaser	16
11. References	16
12. Contact information	16

1. Introduction

ColoNode® (kit and software) is intended 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.

ColoNode® is a test intended for measurement of mRNA expression levels of five biomarker genes, i.e. CEACAM5 (carcino-embryonic antigen), KLK6 (kallikrein-related peptidase 6), SLC35D3 (solute carrier family 35 member D3), POSTN (periostin) and MUC2 (mucin 2), as well as expression levels 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 with high sensitivity and specificity in the same LN 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 genes are expressed in CRC tumor cells, namely CEACAM5, KLK6, SLC35D3 and MUC2, and one, POSTN, is expressed in tumor associated fibroblasts. These five genes have either no or low expression 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 has been 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, 17} 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. 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. CRC tumors that are mucinous (10-20%) 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 has been shown to detect all five biomarker mRNAs and 18S rRNA with equal efficiency in RNA extracted from fresh-frozen, only formalin-fixed and formalin-fixed paraffin-embedded LN tissue.^{9, 17}

1.3 Assay principle

The ColoNode® kit is a dual 3-plex, one-step, real-time, quantitative reverse transcriptase-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 take place in the same reaction mixture with all required reagents added initially, thereby limiting risk for contamination 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 produce 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 the cDNA-bound probe thereby releasing the fluorescent reporter dye, increasing the fluorescence intensity with each PCR-cycle. This process is monitored in real-time and 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 to the ColoNode® Software where it is analyzed. Tumor cell detection is calculated as described in references 8 and 17. Estimation of risk of tumor recurrence is calculated as described in reference 17, i.e. the formula given therein is applied on all analyzed LNs with a CEACAM5 mRNA level (copies/18S rRNA copy) above control cut-off level (9×10^{-9} CEACAM5 mRNA copies/ 18S rRNA copy).¹⁷ The cut-off levels for POSTN and MUC2 that are used in the formula are 2×10^{-5} POSTN copies/18S rRNA copy and 5.95×10^{-2} MUC2 copies/18S rRNA copy, respectively. The limit of quantification (LoQ) is used to determine whether KLK6 and SLC35D3 are included in the risk calculation or not which is 200 copies/ μ L for both biomarkers. In addition, for every sample the mean concentration in copies/ μ L is given for the five biomarker mRNAs and for 18S rRNA.

Number of analyzed lymph nodes = 20

[Valid = 20, Invalid = 0]

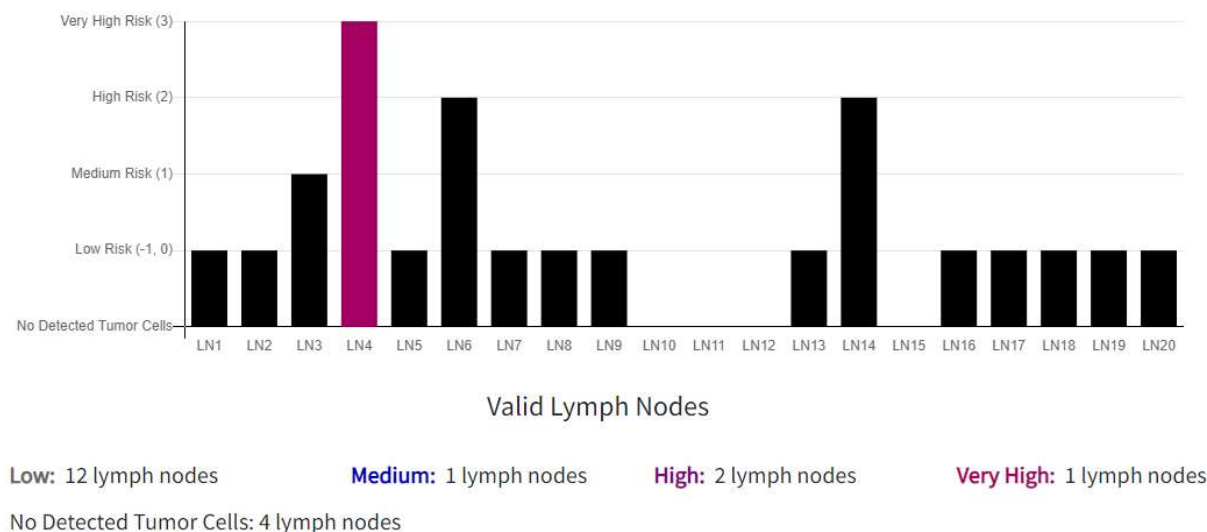


Figure 2: Example of report for a patient classified as a Very High Risk patient by ColoNode®. The risk group value of individual LNs is shown as black bars and the LN with the highest risk group is presented as a colored bar. Lack of a bar indicates that the LN had no detected tumor cells.

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 with two different 3-plex reaction mixes run in parallel. The kit contains two master mixes that each include primers and probes for three of the target genes. It also contains standard samples (STDs) with mixes of three of the target genes in each at defined RNA copy concentrations. Finally, it contains one no template control (NTC) intended for both 3-plex assays. NTC, STDs and 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 target 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 target genes 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 LN samples to generate standard curves. The analyzed samples get the copy number/μl for each target gene extrapolated from the respective standard curve. The RNA copy standards also serve as positive controls of the ColoNode® kit performance. The NTC for each master mix serves as control for contamination. qRT-PCR analysis of 18S rRNA in LN samples serves 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 not be interpreted 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-to-use reagents (according to Table 1) for analysis by qRT-PCR of 20 samples of total RNA extracts of LNs. The kit is delivered as two parts, i.e. one box for each required storage temperature.

Table 1. Content of the ColoNode® kit

Reagent	Tube labelling	No. of tubes	Cap color	Volume (μL/tube)	Storage condition	Box nr
Master mix for system 1	ColoNode® Master mix 1	1	Green	790	-15°C to -25°C	1(2)
Master mix for system 2	ColoNode® Master mix 2	1	Blue	790	-15°C to -25°C	1(2)
Standards (STDs) for system 1	ColoNode® Standard A, Standard B, Standard C	3	Green	10	-70°C to -90°C	2(2)
Standards (STDs) for system 2	ColoNode® Standard D, Standard E, Standard F	3	Blue	10	-70°C to -90°C	2(2)
Manganese acetate (25 mM)	ColoNode® 25 mM MnAc	1	White	400	-15°C to -25°C	1(2)
No template control (NTC)	ColoNode® Negative control	1	Grey	20	-15°C to -25°C	1(2)

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 more information see section 7.7). 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 (ranges 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 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
- 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 no. A28139, Applied Biosystems™). Installation, calibration and maintenance shall be performed according to manufacturer's instructions and recommendations.
- NanoDrop™ (Thermo Scientific™) 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 no. 4314320, Applied Biosystems™).
- Applicator to seal the plastic cover on the 96-well plate (MicroAmp™ Adhesive Film Applicator, Catalog no. 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 (v1.5).
- ColoNode® Software for analysis of ColoNode® kit qRT-PCR raw data from the Quant Studio software. Find ColoNode® Software at <https://software.colonode.se/login> and use the login information supplied on the delivery note.

2.4 Downloads

Information available for download from <https://colonode.se/product> are:

- ColoNode® Instructions for use (IFU)
- Set-up template for ColoNode® qRT-PCR runs on QuantStudio™ 5
- ColoNode® kit 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 thawing-freezing.
- The ColoNode® kit is delivered as two parts, i.e. one box for each required storage temperature.
- Store the ColoNode® Master mixes 1 and 2, Manganese Acetate (MnAc) and Negative control (NTC) at -15°C to -25°C. These are delivered in Box 1(2).
- Store the ColoNode® Standards A-F at -70°C to -90°C. These are delivered in Box 2(2).

4. Precautions and safety

- The ColoNode® 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.
- Do not pool reagents with different kit lot numbers or different tubes of the same lot.
- Do not use damaged reagent tubes.
- Use of this product should be limited to personnel with qualifications in handling of RNA and real-time qPCR analysis.
- When working with the ColoNode® kit always wear a laboratory coat and powder free disposable gloves.
- All frozen components except manganese acetate (MnAc) should be completely thawed on ice and be kept on ice during the entire procedure. MnAc should be thawed and kept in room temperature.
- Avoid microbial contamination and prevent introduction of RNases into kit components and samples.
- Be sure to avoid cross-contamination of the ColoNode® kit when preparing the 96-well plate with master mix and RNA samples. Furthermore, the used equipment should be dedicated to the ColoNode® working area and should not be moved to other working areas.
- 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.
- Dispose all PCR amplified 96-well plates immediately after the qRT-PCR run without opening the plastic cover in order to avoid cross-contamination.
- The ColoNode® kit contains no ingredients at hazardous concentrations. For more information, please read the ColoNode® kit safety data sheet available at our website (<https://colonode.se/product>).
- The ColoNode® kit does not contain any infectious substances or agents, which can cause disease in humans or animals.
- Disposal of kit components should be in accordance with the national biohazard safety guidelines, country, federal, state and local regulations.

5. Procedural limitations

ColoNode®:

- 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 five described biomarkers and 18S rRNA
- can analyze a maximum of 20 LN samples per kit (if one patient has more than 20 LNs several ColoNode® kits should be used)
- should be used for LNs from one patient since the risk of relapse in CRC is calculated from the biomarker expression profile of all lymph nodes in the same analysis

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 the results of the analysis have been checked 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, only formalin-fixed and formalin-fixed paraffin-embedded LN tissue of CRC patients.^{9,17}

6.2 Homogenization and RNA extraction

For large LN tissue volumes such as half LNs, fresh frozen or formalin fixed, the first step is to homogenize the LN tissue by using a method that provides a homogeneous suspension of the entire LN tissue sample. **NOTE!** *It is very important that the entire LN tissue is completely homogenized to achieve representative results.* In the second step, the homogeneous suspension, or an aliquot thereof, is used for extraction of total RNA. The homogenization step is not required for extraction of RNA from 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. Validated methods for extraction of RNA:

- For fresh frozen LNs: extract total RNA by using the acid-guanidinium thiocyanate-phenol-chloroform method.¹⁸
- For formalin fixed LNs: the High Pure FFPE RNA Isolation Kit (Catalog no. 06650775001, Roche) can be used for total RNA extraction from both large tissue volumes such as half LNs and LN tissue sections.¹⁷ For thin sections, the RNeasy DSP FFPE Kit (Catalog no. 73604, Qiagen) can also be used.⁹

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 100 – 800 ng/μL. For concentrations >800 ng/μL dilute the total RNA sample to be within the given interval. Concentrations between 300 – 600 ng/μL, are preferred. 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

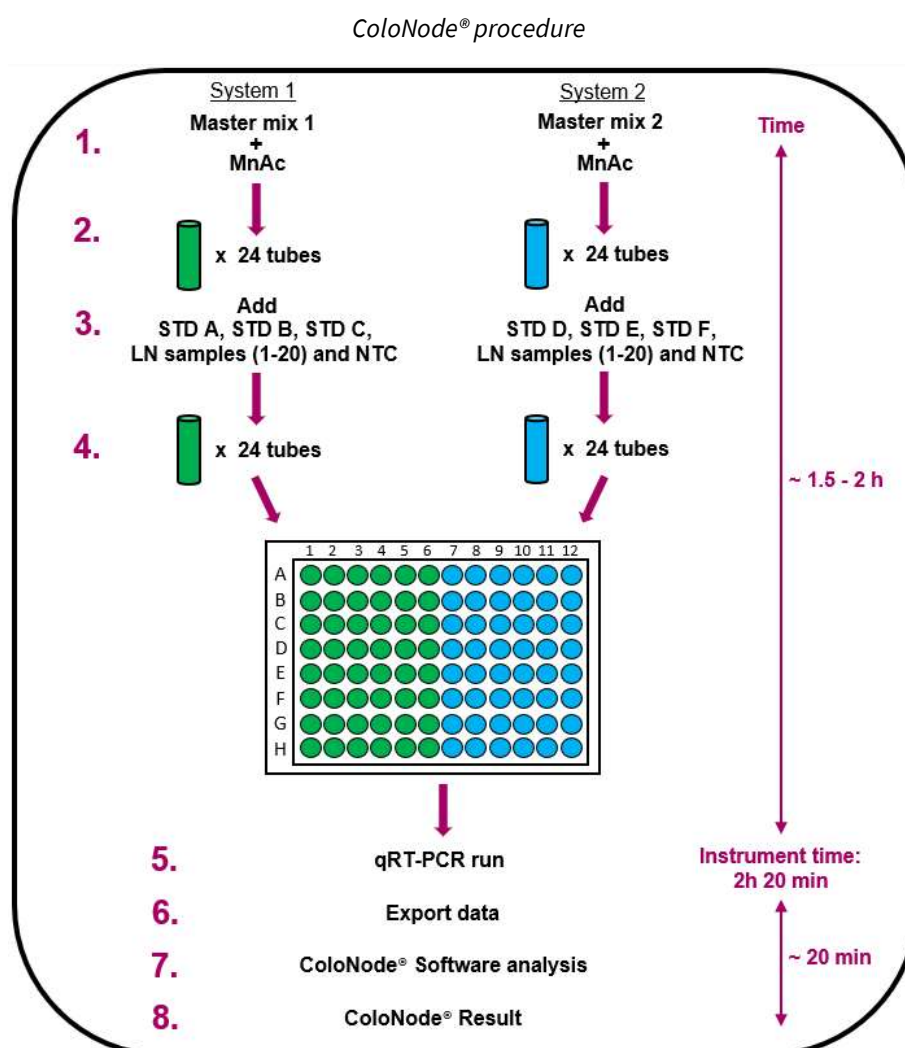


Figure 3. Simplified flowchart procedure of ColoNode® when analyzing 20 LNs. 1) Add MnAc to the master mix tubes for system 1 and 2. 2) Aliquot each master mix up to 24 tubes labelled according to Figure 4. 3) Add RNA to the respective tube, i.e. 3 standards (STDs) unique for system 1 and system 2 (tubes A, B, C and D, E, F, respectively), as well as 1 no template control (NTC) and 20 LN samples per master mix. 4) Load a 96-well plate placing the samples at the positions shown in Figure 5. 5) Run the plate in the qRT-PCR instrument. 6) 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 except for the MnAc tube that should be kept at room temperature.
- Use disposable, sterile and RNase free plastic ware and filter tips.
- Wear disposable powder-free gloves while handling reagents and RNA samples in order to prevent RNase contamination from the skin. Change gloves frequently.
- Proper microbiological aseptic technique should be used when working with RNA.
- Work in an RNase-free area.

7.2 ColoNode® procedure

1. Download the set-up template file for the qRT-PCR run from www.colonode.se/product 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 opening the template file, make a copy and name it with a patient unique code. **NOTE!** *In accordance with the General Data Protection Regulation (GDPR), no personal data may be disclosed.*
3. Open the copy named with the patient unique code, select “**Enter run experiment information**” 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. “**Comments - optional**” can be used for notes.

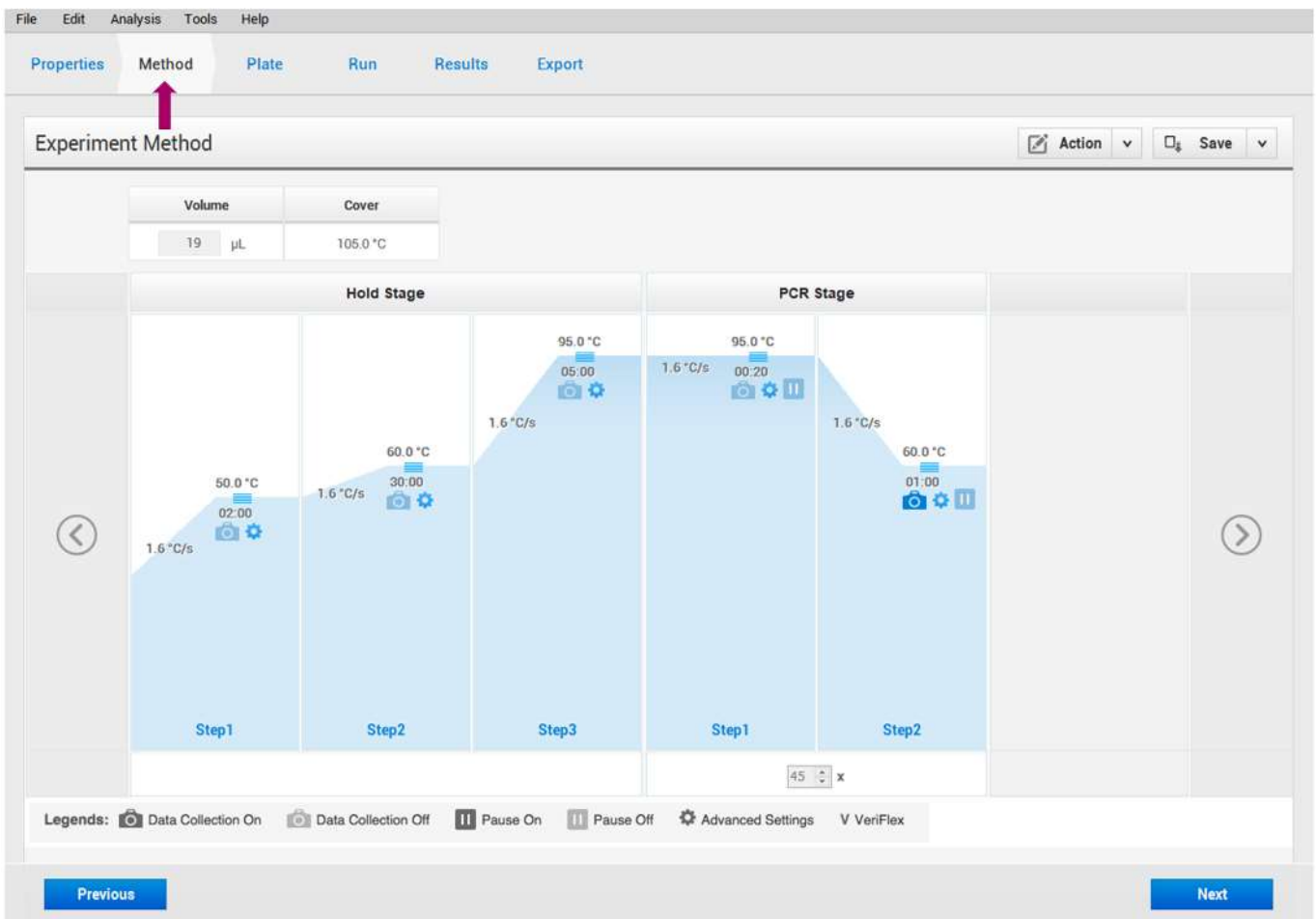
The screenshot shows the 'Experiment Properties' window in the QuantStudio 5 software. The 'Properties' tab is active, as indicated by a red arrow. The window contains the following fields and options:

Name	<input type="text" value="Patient unique code"/>	<input type="text" value="Comments - optional"/>
Barcode	<input type="text" value="Barcode - optional"/>	
User name	<input type="text" value="User name - optional"/>	
Instrument type	QuantStudio™ 5 System	
Block type	96-Well 0.2-mL Block	
Experiment type	Standard Curve	
Chemistry	TaqMan® Reagents	
Run mode	Standard	

At the bottom right, there is a blue 'Next' button. A 'Save' button with a dropdown arrow is located in the top right corner of the window.

QuantStudio™ 5 Design and Analysis Software for qRT-PCR analysis (v1.5)

5. **Optional!** On the upper tab click "**Method**" to view the ColoNode® PCR-profile:



QuantStudio™ 5 Design and Analysis Software for qRT-PCR analysis (v1.5)

- The sample names (STDs, LN samples and NTC) are named in the plate according to figure 5. **NOTE!** Do not change anything in the "**Plate**" tab!
- Prepare tubes (RNase-free, 1.5 mL): three STDs, one NTC and up to 20 LN samples per master mix and label them according to Figure 4 (green and blue coded). Thus, for a full plate totally 48 tubes, 24 tubes per master mix, should be prepared.

Labelling of tubes

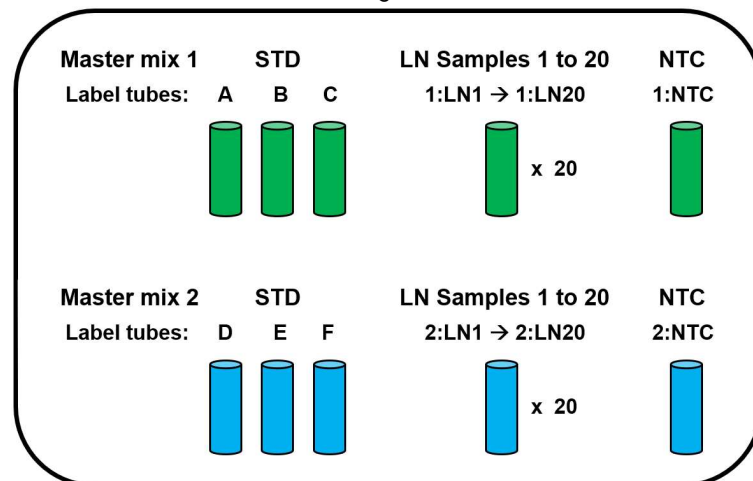


Figure 4. For each master mix: Label tubes for NTC (1:NTC and 2:NTC), three STD concentrations (A, B, C and D, E, F, respectively) and 20 LN samples (1:LN1 to 1:LN20 and 2:LN1 to 2:LN20, respectively), i.e. totally 24 tubes/master mix and 48 tubes for the entire qRT-PCR run.

8. Thaw and keep the MnAc in room temperature. When thawed, mix the MnAc tube thoroughly (~10 sec. high speed several times). **NOTE!** It is very important that the MnAc is completely thawed and mixed before the activation of the Master mixes in step 11 to ensure an effective qRT-PCR run.
9. Thaw the rest of the reagent tubes (Master mix 1 and Master mix 2, NTC and STDs A-F) and keep them on ice. When thawed, invert the Master mix tubes five times and vortex all the other tubes short but thoroughly (~10 sec. high speed). Centrifuge briefly all tubes in a benchtop centrifuge prior to use to get the entire volume of reagent at the bottom of the tube. **NOTE!** It is very important that the STD tubes are mixed properly to obtain approved standard curves.
10. The RNA samples should be thawed and kept on ice. Mix by vortexing (~5 sec.) and centrifuge briefly.
11. Activate the master mixes for system 1 and system 2 by adding 165 μ L MnAc per master mix tube. Invert the master mix tubes several times, vortex the tubes thoroughly (~5 sec.) and centrifuge briefly.
12. Aliquot 37 μ L of activated master mix solution to the 24 tubes labelled for system 1 (green) and system 2 (blue), respectively. Put the tubes on ice.
13. Add 3 μ L of STD or 3 μ L of LN RNA sample or 3 μ L of NTC to the respective labelled tubes with activated Master mix 1 and Master mix 2. Vortex shortly (~5 sec.) and centrifuge down the solution.
14. Distribute the volume from each tube from step 13 by pipetting 19 μ L/well in duplicates in the 96-well plate according to the scheme depicted in Figure 5. Ensure that the samples are entered in the correct wells. If there are fewer than 20 LN samples to analyze, leave the residual wells dedicated for samples empty. The negative controls (NTCs) should always be at the positions shown in Figure 5, i.e. 1:NTC in wells H5–H6 and 2:NTC in wells H11 –H12. If there are more than 20 LN samples from one patient, see sections 5 and 7.7.

Sample positions in the 96-well plate

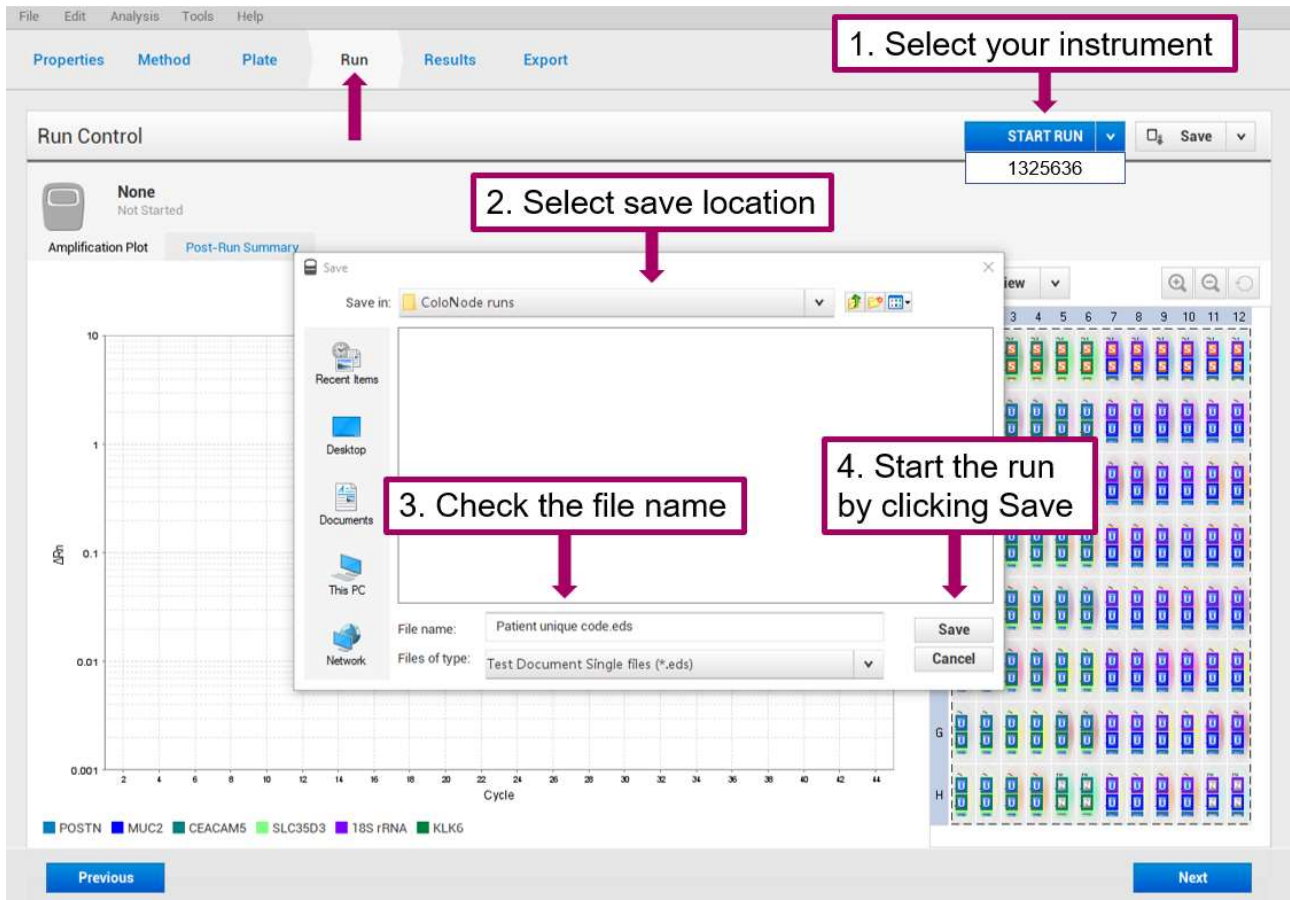
	1	2	3	4	5	6	7	8	9	10	11	12
A	STD A	STD A	STD B	STD B	STD C	STD C	STD D	STD D	STD E	STD E	STD F	STD F
B	1:LN1	1:LN1	1:LN2	1:LN2	1:LN3	1:LN3	2:LN1	2:LN1	2:LN2	2:LN2	2:LN3	2:LN3
C	1:LN4	1:LN4	1:LN5	1:LN5	1:LN6	1:LN6	2:LN4	2:LN4	2:LN5	2:LN5	2:LN6	2:LN6
D	1:LN7	1:LN7	1:LN8	1:LN8	1:LN9	1:LN9	2:LN7	2:LN7	2:LN8	2:LN8	2:LN9	2:LN9
E	1:LN10	1:LN10	1:LN11	1:LN11	1:LN12	1:LN12	2:LN10	2:LN10	2:LN11	2:LN11	2:LN12	2:LN12
F	1:LN13	1:LN13	1:LN14	1:LN14	1:LN15	1:LN15	2:LN13	2:LN13	2:LN14	2:LN14	2:LN15	2:LN15
G	1:LN16	1:LN16	1:LN17	1:LN17	1:LN18	1:LN18	2:LN16	2:LN16	2:LN17	2:LN17	2:LN18	2:LN18
H	1:LN19	1:LN19	1:LN20	1:LN20	1:NTC	1:NTC	2:LN19	2:LN19	2:LN20	2:LN20	2:NTC	2:NTC

← Green: System 1, i.e. samples in Master mix 1
Blue: System 2, i.e. samples in Master mix 2 →

Figure 5. Sample positions in the 96-well plate. The samples added to master mix 1 are placed at the left side (green) and the samples added to master mix 2 are placed at the right side (blue). STD A-C = RNA copy standard for System 1; STD D-F = RNA copy standard for System 2; **NOTE!** Each LN total RNA extract sample is analyzed in duplicate for System 1 (1:LN1 to 1:LN20) and System 2 (2:LN1 to 2:LN20), respectively; 1:NTC = no template control for system 1; 2:NTC = no template control for system 2.

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

19. Open the patient unique setup file (.edt). On the upper tab, click "**Run**". Start the run by clicking on the blue button named "**START RUN**", choose the serial number of QuantStudio™ 5 instrument. Save the run with a patient unique code (.eds) by clicking "**Save**" in the box that appears.

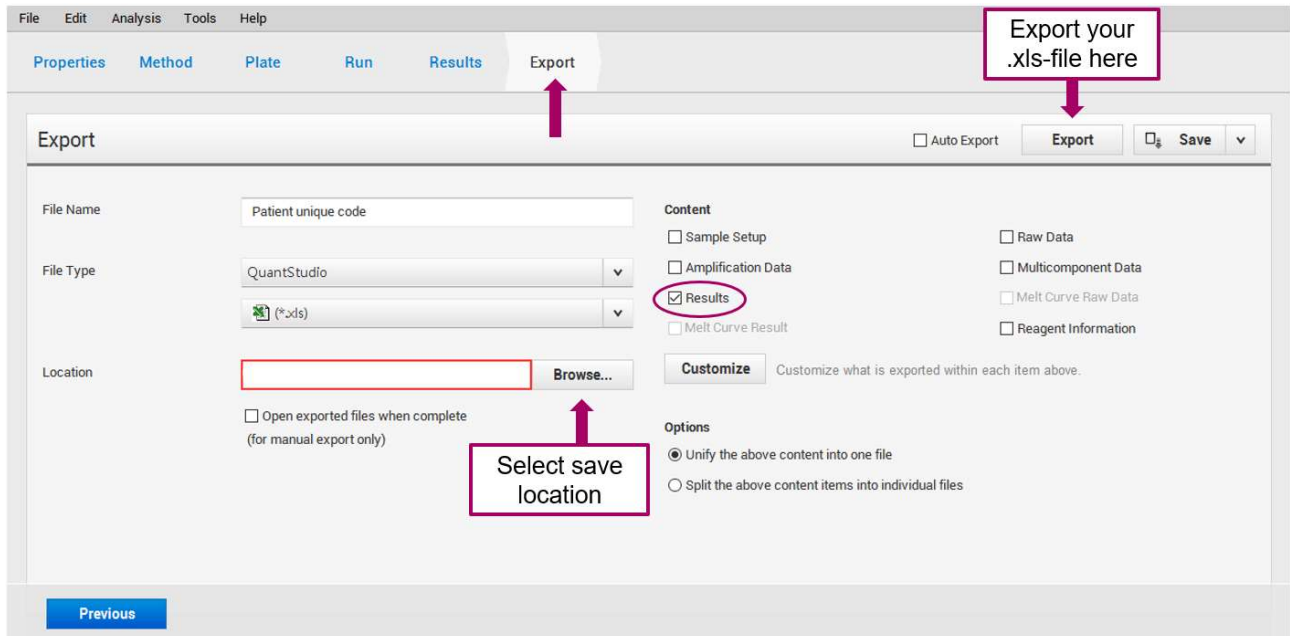


QuantStudio™ 5 Design and Analysis Software for qRT-PCR analysis (v1.5)

20. The run takes approximately 2 hours and 20 minutes.
21. After the run is finished the amplification plots are presented under the tab "**Results**". It is optional to examine the plots. If there were empty wells in the plate, the y-axis range might need to be adjusted (example: minimum value 0.001 and maximum value 10) to get a better view of the plots. False curves and signals from empty wells are excluded from the results by the ColoNode® Software.

7.3 Export of qRT-PCR instrument result

- When the qRT-PCR run is finished, export the raw data as an Excel-file (.xls) by:
 - On the upper tab, click **"Export"**
 - Check that the file name is there otherwise fill it in
 - Under **"Content"**, mark **"Results"**
 - Browse to a preferred **"Location"** for the excel-file on the computer
 - Click **"Export"**











QuantStudio™ 5 Design and Analysis Software for qRT-PCR analysis (v1.5)

- Make a back-up copy of the raw data eds.-file, for eventual troubleshooting in the future.
- Transfer the exported Excel-file (.xls) to a computer that is connected to the internet in order to access the ColoNode® Software at the ColoNode® homepage (<https://software.colonode.se/login>).

7.4 ColoNode® Software procedure


Analysis of results using the ColoNode® Software:

- Log in to the ColoNode® Software by using the details received on the delivery note.
- Upload the exported Excel-file (.xls) from step 3 under 7.3 by clicking **"Select file"** , enter the lot number and click **"Upload file"** .
- Click **"Analyze file"** . When the calculations are ready there will be a green sign saying: "OK Analysis successful". Scroll down to see the ColoNode® result containing:
 - COLONODE TEST RESULT SUMMARY including the overall tumor aggressiveness of the patient, i.e. risk group classification and a barplot of the risk factor for each valid individual LN
 - Tumor cell detection (CEACAM5 level as estimate of tumor cell load in the individual LNs)
 - Risk factor of each individual LN
 - Concentration of biomarker mRNAs and 18S rRNA in copies/μL in the individual LN RNA extracts
 - Summary of quality control of standards and no template controls
 Details can be viewed by pressing the **"Information"** buttons .
 - For more detailed information (Ct- and quantity-values) of each well click **"Show all samples"** . To view this information for individual LN samples click on  in the table "Concentration of biomarker mRNAs and 18S rRNA in copies/μl".
- To save the result click 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. In addition, the concentration of the five biomarker mRNAs and 18S rRNA in each LN can also be saved and downloaded as an Excel file by clicking the green box with an **"Excel file"** symbol .

7.5 ColoNode® Software quality controls

The following aspects will be checked by the ColoNode® Software quality controls and should be interpreted as follows:

✓ Approved	! Not approved (invalid)
No signal in NTCs	Signal(s) in NTC(s). → All results are invalid. Complete re-run is required.
Standards within accepted range	Standard(s) outside accepted range. → All results are invalid. Complete re-run is required.
Ct difference between sample duplicates within accepted range	Ct difference between sample duplicates outside accepted range. Re-run of sample is recommended. Signal in only one of the sample duplicates. Re-run of sample is recommended.
18S rRNA sample concentration within accepted range	Too low 18S rRNA content. Re-run of sample is recommended.

Check all samples by clicking on "**Show all samples**"  to read the causes for potential invalid samples and decide if a re-run of samples is needed.

7.6 ColoNode® result evaluation















The ColoNode® risk group must be evaluated together with other risk factors for tumor recurrence, e.g. lymphovascular invasion and perineural invasion. In addition, the benefit of adjuvant chemotherapy must be assessed taking into account e.g. old age and comorbidity.

ColoNode® risk group	Recommendation of adjuvant chemotherapy
Low	No
Medium	Probably
High	Yes
Very high	Yes

7.7 Interpretation of multiple ColoNode® analyses for the same patient

If the patient has more than 20 LNs more than one ColoNode® kit need to be used and analyzed separately in the ColoNode® Software. For each analyzed LN the results are presented as detected or non-detected tumor cells, and one risk factor (tumor aggressiveness) for relapse in disease, is given to each tumor cell positive LN. The highest risk group from all performed analysis represents the final result of the patient. The same approach is used when interpreting re-runs of samples.

8. Meaning of symbols used on labels

	In vitro diagnostics
	Lot or batch number
	Catalog number
	Stock keeping unit
	Unique device identifier
	Expiry date
 e-instructions	Consult IFU supplied at the home page
	Manufacturer
	Storage temperature
	Keep away from sunlight
	Do not reuse
	Do not use if package is damaged
	Conformité Européenne mark
	Distributor

9. 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
 MnAc, manganese acetate
 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

10. 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.

11. 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. Nicastrì 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. Fahlgrén 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. Lindmark G, Olsson L, Sitohy B, et al. qRT-PCR analysis of CEACAM5, KLK6, SLC35D3, MUC2 and POSTN in colon cancer—An improved method for assessment of tumor stage and prognosis. *Int J Cancer.* 2023;1-12. Doi:10.1002/ijc.34718
18. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. *Anal Biochem.* 1987;162:156-159.

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 ColoNode®.