HANDBOOK

To achieve reliable results, this handbook must be followed precisely.

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1. Introduction

The ColoNode® kit is for research use only.

1.1 Intended Use

The intended use is for detection of tumor cells in colorectal cancer (CRC) lymph node (LN) samples and determination of CRC tumor cell aggressiveness.

The ColoNode® kit is a test intended for measurement of mRNA expression levels of five genes: CEACAM5 (carcinoembryonic antigen), KLK6 (kallikrein-related peptidases 6), MUC2 (mucin 2), SLC35D3 (solute carrier family 35 member D3), POSTN (periostin) and 18S rRNA (18S ribosomal RNA), in total RNA extracted from CRC LN samples.

1.2. Background

Biomarker mRNAs have great potential as tools for LN analysis in CRC. Analysis of biomarker expression at the mRNA level has several advantages over protein level or cellular level expression: several biomarkers can easily be analyzed in the same RNA extract and a large volume of the LN (potentially the entire LN) can be available for analysis.[1]

In the ColoNode® kit six target genes are analyzed. Four of the genes are expressed in CRC epithelial cells, namely CEACAM5, KLK6, SLC35D3 and MUC2, and one biomarker, POSTN, is expressed in tumor associated fibroblasts. None of the genes are expressed in immune cells making them suitable for analysis of 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.[2] CEACAM5 is expressed in epithelial cells of the large bowel and their expression is retained in CRC.[3] CEACAM5 mRNA has been shown to be superior in detection of disseminated tumor cells in CRC LNs compared to the routine method (hematoxylin and eosin staining of LN tissue).[3-4] Moreover, CEACAM5 mRNA level have been shown to correlate with numbers of tumor cells.[4]
- 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.[5] KLK6 mRNA has been identified as a promising progression biomarker for CRC. High levels of KLK6 mRNA correlates to poor prognosis.[6]
- Mucin is the major mucin in the mucous layer covering the colon and rectum epithelium. Ten to 20% of all CRC tumors are mucinous, which involves copious production of intestinal mucins, mainly MUC2.[3] Mucinous primary tumors have been shown to be associated with better prognosis than adenocarcinoma in general.[7]
- POSTN is expressed in tumoral stromal fibroblasts in CRC. The level of stromal POSTN is a prognostic biomarker for CRC.[8]
- SLC35D3 is analyzed because it is associated with poor prognosis and appears to represent another epithelial cell expression pattern than CEACAM5, MUC2 and KLK6 perhaps related to cellular immaturity.[9]
- 18S rRNA is used for normalization of mRNA expression levels in analysis of different genes and samples. 18S rRNA has been proven to be expressed at relatively stable levels in lymphocytes and colonic epithelial cells.[10]

The biomarker combination of ColoNode® was able to detect tumor cells and an estimated grade tumor cell aggressiveness in CRC from fresh frozen LN samples. Moreover, the ColoNode® kit could be used successfully for analysis of formalin-fixed LN sections.[Unpublished results 2020]

1.3 Assay principle

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

In a 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 possible because the enzyme, Tth DNA polymerase, functions both as a reverse transcriptase and a DNA polymerase.

Starting material is total RNA, which is reverse transcribed to cDNA with a 5'-gene specific (reverse) primer that binds to the mRNA of the respective gene of interest and produces the specific cDNA. The amplification step utilizes a pair of primers (forward and reverse) complementary to one strand each and a gene 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. As long as the probe is intact, it will not emit fluorescence. As the elongation of the strand proceeds the probe will be cleaved by the Tth DNA polymerase releasing the reporter dye, and as the amplicons increase exponentially with each PCR cycle, fluorescence signals increase and are detected in real-time. When the intensity of the particular fluorescence signal reaches a pre-defined threshold, the corresponding cycle is referred as the cycle threshold (ct).

The ColoNode® kit raw data results from the qRT-PCR analysis is transferred from the QuantStudio™ 5 Real-Time PCR System and analyzed by the ColoNode® Software yielding tumor cell detection and estimation of CRC tumor cell aggressiveness. In addition, expression levels of each target gene mRNA for every analyzed sample is given.

Figure 1 – Overview of ColoNode® analysis

1.4 Assay design
The ColoNode® kit is designed as two 3-plex, one step, qRT-PCR systems.

The kit performs the reverse transcription (RT), amplification and detection of mRNA by qRT-PCR with two 3-plex reaction mixes. It contains two master mixtures with primers and probes for three target genes each. It also contains standard samples for actual gene expression value determination (copies/µl) and negative control. Unknown LN samples, standards and negative control are analyzed in duplicate.

Specific reverse and forward primers have been constructed for each of the six target genes. They are placed in different exons of respective target gene. A sequence-specific dye-labeled probe is placed over the boundary between the two exons to make the assay RNA specific and thereby avoiding amplification of genomic DNA. Three different fluorescence dyes (FAM™, VIC™, NED™; Thermo Fisher Scientific Inc) are used to perform quantification of 3 target genes in a single reaction. Three genes are measured in half of the wells on the plate and the other 3 genes are measured in the other half.

In each analysis, standard samples containing specific amounts of mRNA from each of the 6 target genes are analyzed and form a standard curve. The analyzed unknown samples get the actual gene quantity per µl calculated from the respective target gene standard curve.

Both the standards and the analysis of the reference gene 18S rRNA function as a positive control for the test and the unknown samples. The 18S rRNA gene also verifies that enough amounts of total RNA have been analyzed. A negative control is included for control of contamination.

2. Material and Equipment
The ColoNode® kit contains ready for use reagents for analysis of 20 LN RNA samples, 6 standard samples and 2 negative controls.

Note! The kit shall be used for a maximum of 20 LN samples. Use additional kits if more than 20 LNs need to be combined. The result of the detected tumor cells for each sample will be presented and one estimated tumor cell aggressiveness factor will be presented per analyzed kit and plate. The highest tumor cell aggressiveness factor should then represent the result if several plates needs to be combined, i.e. 2 plates are analyzed from the same origin.

2.1 Content

Table 1 - Content of the ColoNode® kit

Reagent	Tubes /kit	Cap colour	Volume (µl)	Storage condition
ColoNode Mastermix 1	1	Green	830	-20ºC
ColoNode Mastermix 2	1	Blue	830	-20ºC
ColoNode Standard 1 tube 1-3	3	Green	5	-80ºC
ColoNode Standard 2 tube 1-3	3	Blue	5	-80ºC
Manganese Solution	1	White	300	-20ºC
Negative control	1	-	20	-20ºC

2.2 Materials required, but not provided

General laboratory equipment
The following general laboratory equipment is required to perform ColoNode® kit analysis. All laboratory equipment must be installed, calibrated, operated and maintained according to the manufacturer's instructions and recommendations.
- 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
- Plate centrifuge capable of PCR-plates

General laboratory consumables and reagents
The following general laboratory consumables and reagents are required to perform ColoNode® kit analysis:
- 1.5 ml tubes for PCR-use
- Pipette tips with aerosol barrier for PCR-use
- RNaseZap™ RNase Decontamination Solution or equivalent

Recommended special equipment, reagents and consumables
- QuantStudio™ 5 Real-Time PCR System, 96-well, 0.2 ml (Standard block, catalog #A28139, Applied Biosystems™). Installation, calibration and maintenance shall be performed according to manufacturer's instructions and recommendations.
- Homogenization and RNA extraction reagents used for preparation of total RNA
- NanoDrop or equivalent 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™) or equivalent
- Applicator to seal the plastic cover on the 96 well plate (MicroAmp™ Adhesive Film Applicator catalog #4333183, Applied Biosystems™)

2.3 Softwares
- QuantStudio™ Design and Analysis Software v1.5.1QS5 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
Supplementary information can be downloaded from www.colonode.se. The following items are available:
- ColoNode® kit Handbook
- ColoNode® brochure
- Set up template for ColoNode® kit analysis on QuantStudio5™
- ColoNode® chemical information data sheet

3. Storage requirements

- The ColoNode® kit reagents are stable until the expiration date when stored and handled as described in this handbook.

- 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 single use only.

- Avoid repeated freezing-thawing.

- Store the ColoNode® kit components according to following:
  - Mastermixes, manganese acetate (MnAc) and negative control at -20ºC (=4 tubes)
  - Standard samples at -80ºC (=6 tubes)

4. Precautions and safety

- The kit procedure should be performed according to this handbook.

- Deviations from this handbook can compromise the kit performance and results.

- It is important to follow the instructions when analyzing the results in QuantStudio5, otherwise the ColoNode Software results can be compromised.

- The ColoNode® kit contains no hazardous ingredients. For more information please read the ColoNode® safety data sheet available at our website (www.colonode.se).

- Use of this product should be limited to personnel with qualifications in RNA handling and real-time qRT-PCR analysis.

- When working with the ColoNode® kit always wear a laboratory coat and powder free disposable gloves.

- Do not pool reagents with different kit lot numbers or different vials of the same lot.

- Do not use damaged reagent vials.

- Frozen components should be completely thawed on ice and be used directly when thawed.

- 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.

- Highly recommended to keep strict separation when preparing ColoNode® Mastermix, RNA samples and PCR runs in the laboratory to avoid cross-contamination. Furthermore, all laboratory equipment should be dedicated to each area and not be moved between the different working areas and gloves should be changed between the different steps.

- Dispose all PCR amplified plates without opening immediately after the PCR run to avoid cross-contamination.

- Keep working areas clean and free from RNAses by using RNAseZap or equivalent.

- 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 for research use only
- is developed for analysis of CRC LN tissue
- results might be affected if the input RNA quality and concentration deviates from the recommendations in the handbook
- will only measure the gene expression of the described six target genes
- is intended for analysis of a maximum of 20 LN samples per kit

6. Sample requirements

The purity and concentration of sample input RNA is crucial for successful use of the ColoNode® kit.

6.1 Samples
The ColoNode kit has been tested using human total RNA extracted from one half fresh frozen CRC LNs and formalin-fixed CRC LN sections.

6.2 Homogenization and RNA extraction
For fresh frozen tissue 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 of the LN sample is used for extraction of total RNA. The homogenization step can be excluded if formalin-fixed LN tissue sections are used for the RNA extraction. 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 is to extract total RNA by using the acid guanidine phenol chloroform method. [11]
- For formalin-fixed tissue sections is to extract total RNA by using the RNeasy DSP FFPE Kit (Catalog no: 73504, Qiagen).

6.3 RNA purity and concentration
Total RNA concentration and purity should be determined by a NanoDrop or equivalent for each sample. The total RNA for samples should be within the interval of 10 – 1200 ng/µl. Dilute the total RNA to be within interval (only if necessary if concentration is > 1200 ng/µl). For the dilution use RNAse free water with RNAsin added (2,5µl RNasin inhibitor (40U/µl) per 100µl RNase free water). Concentrations between 500 – 1200 ng/µl), are to be preferred. RNA samples should be free from potential PCR inhibiting factors. The ratio A_{260}/A_{280} is used as a measurement of the RNA purity. The recommended value is between 1.8 to 2.1.

7. Instructions for use

7.1 ColoNode® kit procedural guidelines
- Perform all steps on ice.
- Use disposable, individually wrapped, sterile plastic ware and sterile, disposable RNase-free tips and tubes.
- Wear disposable powder free gloves while handling reagents and RNA samples to prevent RNase contamination from the skin; change gloves frequently.
- Always use proper microbiological aseptic techniques when working with RNA.
- It is recommended to use RNaseZap™ RNase Decontamination Solution to remove RNase contamination from work surfaces and from non-disposable items such as centrifuges and pipettes used during PCR setup.

7.2 ColoNode® kit procedure
1. Download the setup (www.colonode.se) for the qRT-PCR run to a USB stick or direct to the PC-computer coupled to the QuantStudio™ 5 Real-Time PCR System.
2. Prepare tubes by labelling one RNase free tube for NTC, one tube per STD concentration (tube 1-3) and one tube per unknown RNA sample to be analyzed (Full plate → 24 tubes per mastermix = 48 tubes in total; Figure 3).
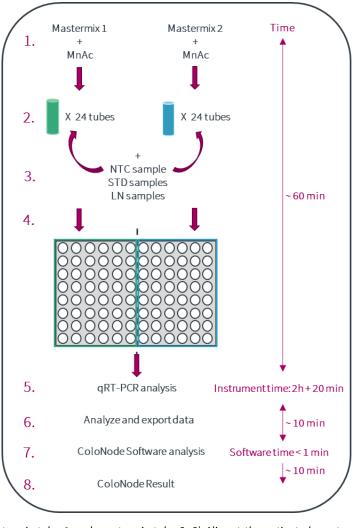
Figure 2 – *Overview of ColoNode® kit procedure*

Figure 2: *1) Add MnAc to the mastermix tube 1 and mastermix tube 2, 2) Aliquot the activated mastermix solutions in 24 1,5ml tubes per activated mastermix, 3) Add the RNA to respective tube (1 negative control (NTC), 3 standard (STD) and 20 unknown LN samples per mastermix), 4) Distribute to 96 well plate, 5) Run the plate in qRT-PCR instrument, 6) Analyze and export data, 7) Use the exported file as input for the ColoNode Software and analyze data, and 8) Receive ColoNode result.*
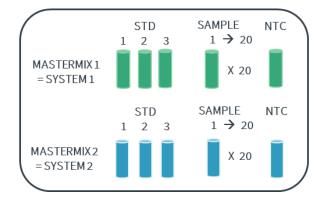
Figure 3 – Labelling of tubes

Figure 3. *Label tubes for 1 (one) NTC, 3 (three) STD and 20 samples per mastermix (24 tubes; n=48 tubes for both mastermixes).*

3. Thaw all reagent tubes and keep them on ice (Mastermixes, MnAc, NTC and STDs).
4. Centrifuge briefly in a benchtop centrifuge prior to use to get the entire volume of reagent at the bottom of the tube.
5. Add 124μl MnAc to each mastermix tube (1 and 2). Vortex shortly (~5s) and centrifuge down the solution.
6. Aliquot 37μl of activated mastermix solution per tube. Put the tubes on ice.

7. Add 3µl of STD, unknown RNA samples or NTC to the labelled tubes with activated mastermix. Vortex shortly (~5s) and centrifuge down the solution.
8. Add 19,5µl of the solution (activated mastermix + RNA) in 2 wells each per sample (STD, unknown samples and NTC) to the 96 well qRT-PCR plate according to following protocol (figure 4):

Figure 4 – Sample placement on 96 well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 Tube 1	STD 1 Tube 1	STD 1 Tube 2	STD 1 Tube 2	STD 1 Tube 3	STD 1 Tube 3	STD 2 Tube 1	STD 2 Tube 1	STD 2 Tube 2	STD 2 Tube 2	STD 2 Tube 3	STD 2 Tube 3
B	Sample 1	Sample 1	Sample 2	Sample 2	Sample 3	Sample 3	Sample 1	Sample 1	Sample 2	Sample 2	Sample 3	Sample 3
C	Sample 4	Sample 4	Sample 5	Sample 5	Sample 6	Sample 6	Sample 4	Sample 4	Sample 5	Sample 5	Sample 6	Sample 6
D	Sample 7	Sample 7	Sample 8	Sample 8	Sample 9	Sample 9	Sample 7	Sample 7	Sample 8	Sample 8	Sample 9	Sample 9
E	Sample 10	Sample 10	Sample 11	Sample 11	Sample 12	Sample 12	Sample 10	Sample 10	Sample 11	Sample 11	Sample 12	Sample 12
F	Sample 13	Sample 13	Sample 14	Sample 14	Sample 15	Sample 15	Sample 13	Sample 13	Sample 14	Sample 14	Sample 15	Sample 15
G	Sample 16	Sample 16	Sample 17	Sample 17	Sample 18	Sample 18	Sample 16	Sample 16	Sample 17	Sample 17	Sample 18	Sample 18
H	Sample 19	Sample 19	Sample 20	Sample 20	NTC	NTC	Sample 19	Sample 19	Sample 20	Sample 20	NTC	NTC

Figure 4. *Recommended sample placement in 96 well plate. The left side represents system 1 (green) and the right side represents system 2 (blue).*

9. Seal the plate with a plastic cover according to QuantStudio5 manual.
10. Centrifuge the 96 well plate briefly in a benchtop centrifuge for 15 to 30 seconds.
11. Put the 96 well plate in the machine.
12. Start the downloaded setup.
13. Check that the following PCR-profile is prepared:

Step 1. 2min 50°C
Step 2. 30min 60°C
Step 3. 5min 95°C
Step 4. 20sek 95°C, 1min 60°C (x 45cycles)

14. Start the run by pressing **START RUN**. The run takes approximately 2 hours and 10 minutes.

7.3 Instrument result analysis
1. When the run is finished start with saving the results before changing parameters (= run raw data).
2. Make a copy of the run raw data and open this file with the software QuantStudio™ Design & Analysis on the computer.
3. Ensure that the samples are entered in the correct wells on the plate.
4. On the upper tab, press **RESULTS**.
5. Press the settings symbol which is located on the right side of the blue button named **ANALYZE**.
6. The instrument has an automatic threshold. This shall be changed by selecting each target one by one and then press unselect **DEFAULT SETTINGS** and **AUTOMATIC THRESHOLD**.
7. Change each target gene to the appropriate threshold by pressing on each respectively and enter the following numbers alongside **THRESHOLD** under **CT SETTINGS FOR TARGET**:
8. To save the changes, press **APPLY**.
9. Press **ANALYZE**.
10. Ensure that the run has been successful from a technical point of view (for more information and support download the support manual; www.colonode.se/downloads):
    1. Ensure that the NTCs are negative in each well for system 1 and system 2.
    2. Mark all STD wells for system 1 and look at the **AMPLIFICATION PLOTS** for each target gene. Three positive curves with duplicates should be seen.
    3. Repeat the same procedure (no 1-2) for system 2.
    4. Ensure that positive samples have duplicates with small variation between curves. If not note these sample numbers.

Table 2 – Target gene thresholds

TARGET GENE	Gene code	THRESHOLD
SLC35D3	M101	0,03
POSTN	M102	0,01
KLK6	M103	0,02
MUC2	M104	0,02
CEA	M105	0,02
18S rRNA	M106	0,01

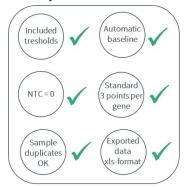*Figure 5 - Summary of the instrument result analysis*

Figure 5. *Summary of the instrument result analysis to be done as described above before export of data.*

11. Export xls-file by:
    Press **EXPORT**
    Under Content, mark **RESULTS**
    Choose a location for the excel file on the computer next to **LOCATION**
    Press **EXPORT**

7.4 ColoNode® Software procedure
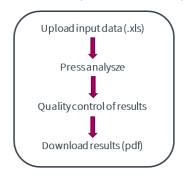
Figure 6 - Summary of the result analysis

Figure 6. *Summary of the result analysis.*

Result analysis with ColoNode® software:
1. Log in to ColoNode® Software by using the details received on the delivery note.
2. Upload the analyzed file from step 11 in previous section (.xls) by pressing **SELECT FILE**, typing **LOT NUMBER** and pressing **UPLOAD FILE**.
   Note! *Not raw data. The analyzed file with correct settings as described in figure 5.*
3. Press **ANALYZE FILE** on the uploaded file and wait while the software calculates the result and shows **SUCCESSFUL ANALYZE** (in green).
4. Scroll down to see the ColoNode® results, Quality control and Quantity mean:
   - ColoNode test result summary: 1. Tumor cell detection
                                                          2. Grade of tumor cell aggressiveness

   - Tumor cell detection per sample
   - Quality controls
   - Quantity mean (copies/μl) per sample for each target gene
5. Check the quality controls:

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

   !   Press on the **INFORMATION** button for this/these sample. Read the reasons for the
          warning and evaluate if a new re-run is needed or if the results still can be used.
6. To save the result press **DOWNLOAD**. All the results will then be saved in a pdf-file.

8. Meaning of symbols used on labels

IVD In vitro diagnostics

LOT Lot or batch number

REF Catalogue number

Expiration date

Manufacturer

9. NOTICE TO PURCHASER

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

10. References

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11. Contact information

The ColoNode® kit and software are manufactured by:
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