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Basic Evaluation of the automated Gene Amplification Detector RD-200 and LYNOAMP CK19

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One-Step Nucleic Acid Amplification (OSNA) is a method that allows the diagnosis of lymph node metastasis and uses a specially designed device and reagents for amplification and detection of cytokeratin (CK) 19 mRNA, which is present only in lymph nodes carrying metastasis. The first OSNA system, developed by Sysmex Corporation (Kobe, Japan), consists of the automated Gene Amplification Detector RD-100i and LYNOAMP BC and it is currently used by several hundred institutions around the world, mainly for breast cancer-related diagnosis.

Now, Sysmex Corporation has developed a new OSNA system, consisting of the automated Gene Amplification Detector RD-200 and LYNOAMP CK19 which allows the analysis of more samples, has shorter turnaround time when compared to the first generation OSNA, and is a multi-application platform. The clinical and analytical performance of this new system was evaluated and results are shown here. Lymph nodes of breast cancer patients were analyzed by using both the conventional and the new system, and concordance rates were calculated; the findings showed that both systems were comparable in clinical performance. Furthermore, the new system has an improved usability which reflects the market needs and leads to facilitated diagnosis with simplified operation and shorter turnaround time in the clinical setting. Due to these elevated improvements, the new system is expected to achieve a broader market acceptance and spread than the first-generation OSNA system.

Key Words OSNA, RD-200, RD-210, LYNOAMP CK19, LYNOAMP CK19 E, Cytokeratin 19 (CK19)

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INTRODUCTION

One-Step Nucleic Acid Amplification (OSNA) is a method for the diagnosis of lymph node metastasis. A specially designed device and reagent amplifies and detects the target gene, cytokeratin (CK) 19 mRNA, present in metastatic lymph nodes. (***Fig. 1***)

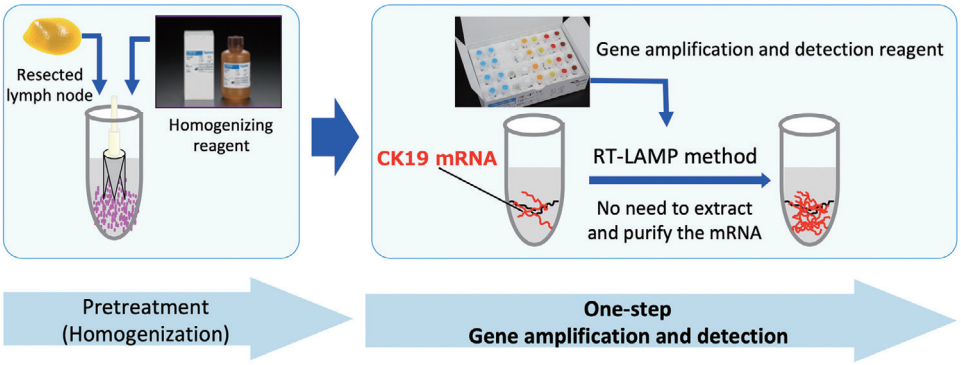

Fig. 1 *OSNA (One-step nucleic acid amplifyication) method*

Notes: This article is based on current regulatory requirements in Japan. (as of Oct. 2018)
The specifications, performances and functions described here may be different depending on the regions or the countries due to the regulatory affairs, legal matters or local guidelines. For more details, please contact your regional affiliates or distributors.
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LYNOAMP BC (Sysmex Corporation, Kobe, Japan) is a reagent kit for the detection of CK 19 mRNA in metastatic lymph nodes of breast cancer patients, which was first approved in 2008 by the Japanese regulatory agency(MHLW: Ministry of Health, Labour and Welfare). (Acceptance of the automated Gene Amplification Detector RD-100*i* as a medical device was made in 2006.) Later, LYNOAMP BC was also approved for colorectal cancer (2010), gastric cancer (2012), and non-small cell lung cancer (2015), and since then this reagent kit has been in use by several hundred institutions worldwide. Though each cancer type is unique from the clinical perspective, the diagnostic approach as well as treatment and the number of lymph nodes to be analyzed is often different among different cancer types. For some cancers, such as colorectal cancer, the number of nodes required for staging is higher when compared to other types in which the sentinel lymph node (SLN) concept is well established. Therefore there is the need for a diagnostic tool that allows the simultaneous analysis of high sample throughput, reduction of workload, and reduced turnaround time, all while minimizing the risk of human error.

To meet such market needs, Sysmex Corporation has further developed the conventional system (model RD-100*i* and LYNOAMP BC) and improved it in terms of performance and usability. The new generation OSNA combines the RD-200 Gene Amplification Detector and LYNOAMP CK19 and was introduced to the Japanese market in July 2018. In Europe, another system that is compatible with the new system, the automated Gene Amplification Detector RD-210 and LYNOAMP CK19 E, was already introduced to the market in July 2017 and has been in clinical use since then.

In the following paragraphs, the new system will be described in detail.

SUMMARY OF PRODUCT

1. Specification and Characteristics of the Reagents

Specification of the new reagent set is shown in ***Table 1***. The new LYNOAMP CK19 reagent kit was improved by reducing the number of tests to 120 per package and by extending the open stability to 2 months. The conventional LYNOAMP BC reagent kit holds 240 tests per package and has an open stability of only 1 month. This allows more flexible management of LYNOAMP CK19 in institutions handling lower numbers of cases and reduces reagent waste. Furthermore, with the 2D bar-code reagent management features, input of data such as the lot number and the number of freezing/ thawing cycles as well as the

Table 1 *Specifications of the new reagent and the conventional reagent*

	Conventional reagent	New reagent
Product name	LYNOAMP BC	LYNOAMP CK19
Intended use	Detection of CK19 mRNA in lymph nodes of resected breast cancer, colorectal cancer, gastric cancer or non-small cell lung cancer (to diagnose lymph node metastasis in breast cancer, colorectal cancer, gastric cancer or non-small cell lung cancer).	Detection of CK19 mRNA in lymph nodes of resected breast cancer, colorectal cancer or gastric cancer (to diagnose lymph node metastasis in breast cancer, colorectal cancer or gastric cancer). *As of September 2018.
Storage	-25 ~ -15°C	-25 ~ -15°C
Shelf-life	12 months	12 months
Expiration date after opening	1 month	2 months
Packaging unit	240 tests	120 tests (LAC-701A)
	CK19 primer solution: 720 µL x 8 vials Enzyme solution: 450 µL x 2 vials CK19 positive control: 110 µL x 4 vials CK19 negative control: 110 µL x 4 vials Calibrator level 1: 110 µL x 4 vials Calibrator level 2: 110 µL x 4 vials Calibrator level 3: 110 µL x 4 vials	CK19 primer solution: 1,400 µL x 3 vials Enzyme solution: 450 µL x 1 vial CK19 positive control: 110 µL x 3 vials Negative control: 110 µL x 3 vials CK19 calibrator level 1: 110 µL x 3 vials CK19 calibrator level 2: 110 µL x 3 vials CK19 calibrator level 3: 110 µL x 3 vials

management of onboard reagent quantity can be done automatically, saving time during sample preparation and reducing the risk of data transcription errors. (***Fig. 2***)

The new reagents come in a redesigned vial, the RD sample vial (Sysmex Corporation), which has improved features and solves operability problems such as minimizing the risk of reagent contamination. (***Fig. 3***)

In the new system, the reagent composition has been improved to increase the amplification efficiency of the target gene and reduce the time required for analysis. Consequently, new cut-off values have been established for this system. Detailed data is shown in the following paragraphs.

Since there were some reported cases with the conventional system in which gene amplification reaction was inhibited by components derived from the tissue specimens [1)], a 10-fold diluted sample and a measurement sample were both measured as reference. The modified reagent of the new system has been improved to minimize the inhibitory effect of contaminants on the gene amplification, therefore the co-measurement of 10-fold diluted samples is not necessary anymore. This was verified in clinical studies that the new system can determine with high accuracy the presence or absence of lymph node metastasis by using only undiluted measurement samples. So, the new system eliminates the additional preanalytical steps which were necessary with the conventional system, reduces the time required for sample preparation, and offers simplified procedures including improved operability and a shorter turnaround time (TAT) for the entire lymph node examination.

2. System Features

1) Space-Saving Design

The RD-200 device can be operated by using the integrated touchscreen only; it does not require any additional device such as laptop or computer. In comparison to RD-100*i* detector, this improvement has achieved space saving of an installation space in addition to increasing the number of samples that can be simultaneously measured up to 14 samples. (***Fig. 4***)

Fig. 2 *2D bar-code reagent management*

Fig. 3 *New vial (RD sample vial) dedicated for the OSNA method*

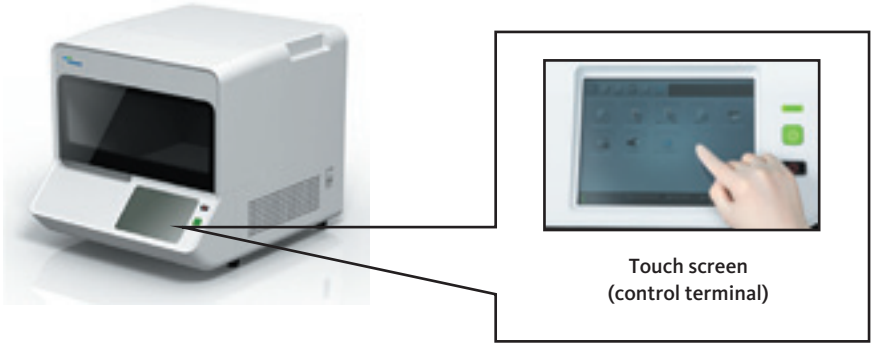

Fig. 4 *Exterior of RD-200 detector*

Furthermore, the RD-200 pipette tip disposal bag can be accessed from the front of the analyzer, unlike the standard side access on RD-100*i*. Since the new OSNA system does not need additional space adjacent to the device for tip disposal, this enables a more efficient use of laboratory work space.

2) Enhanced Internal Quality Control

On the internal quality control screen, the measurement of positive control data (rise time and quantitative values of CK19 mRNA) and the slope of the calibration curve are plotted on a graph in real time. The positive control mean, 2SD and 3SD are automatically calculated and displayed on the graph, thereby allowing the operator to check the changes of the reagent and device conditions.

Furthermore, the new system is capable of automatically storing quality control data of all past lots, which allows the operator to compare the performance of a new lot with the previous lots for better quality assurance. This in contrast to the conventional system, which is capable of storing only the data of the previous lot. (***Fig. 5***)

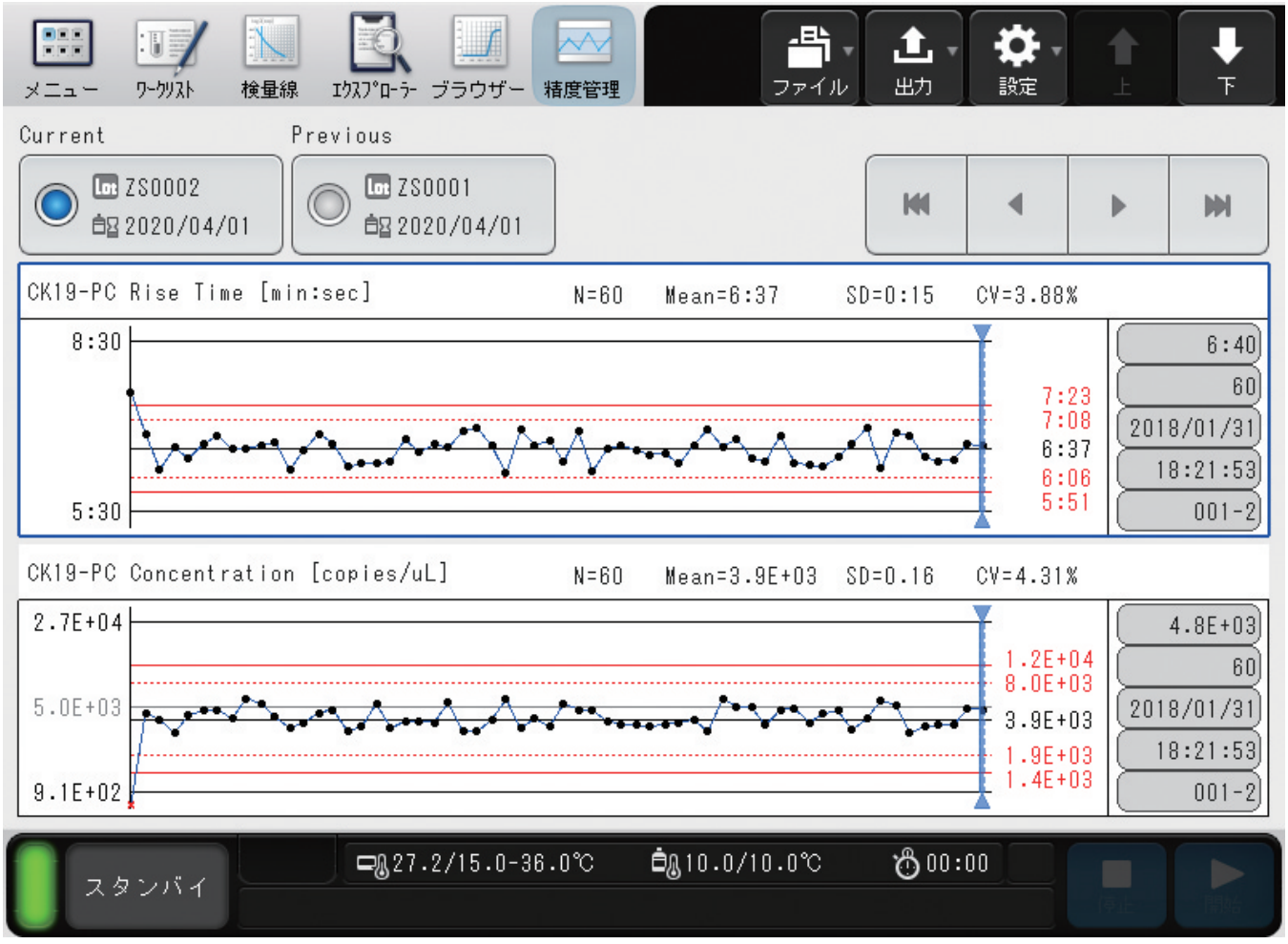

Fig. 5 *Internal quality control screen*

3) Measurement Time (Comparison between RD-100i and RD-200)

The analytical measurement time, which is defined as the time interval between pressing the START button and receiving the result on the display, is shorter for RD-200 than for RD-100*i*. The test results comparing the analytical measurement time between the two detectors is shown below.

1. Materials and Methods

1.1. Target specimen: negative control

1.2. Number of specimens: The number of specimens listed below were measured in a single batch (one each of RD-100*i* and RD-200 were used)

Number of specimens tested with the conventional system: 1, 2, 3 and 4

Number of specimens tested with the new system: 1, 2, 3, 4 and 14

2. Result

When assessing simultaneously 1 to 4 specimens, the measurement time was 5 to 10 minutes shorter with RD-200 than with RD-100*i*, and the Δ time increased proportionally with the number of specimens (***Table 2***). Moreover, the RD-200 allows the analysis of 14 specimens in approximately 30 minutes, and this feature offers the possibility to analyze lymph nodes from one or more patients at the same time as well as use a detector in the intraoperative setting where rapid diagnosis is an essential requirement. (***Fig. 6***)

Table 2 *Test results*

Number of specimens	RD-100*i*	RD-200
1	22 min 18 sec	16 min 58 sec
2	24 min 38 sec	17 min 09 sec
3	26 min 46 sec	18 min 58 sec
4	29 min 17 sec	19 min 19 sec
14	—	29 min 50 sec

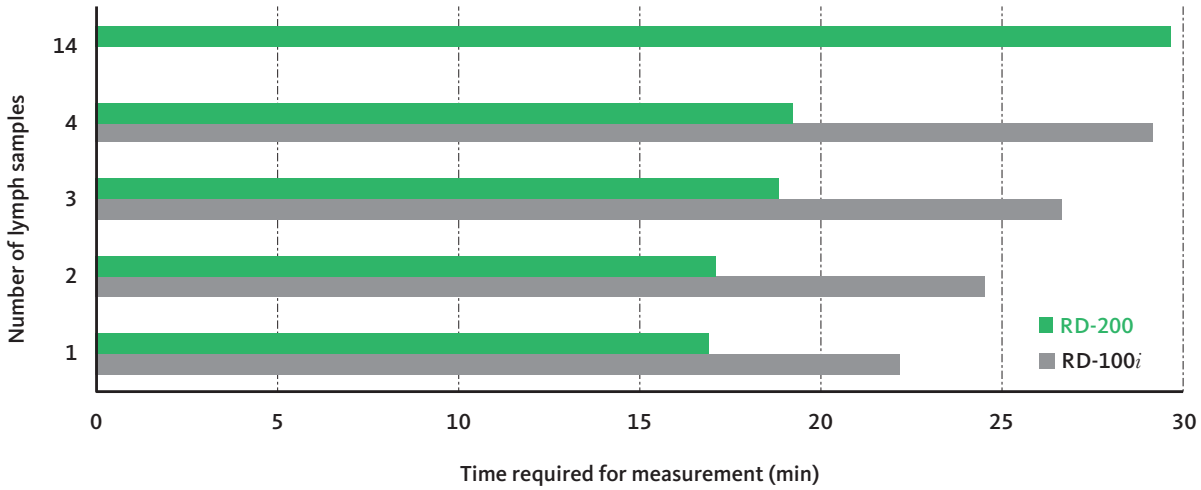

Fig. 6 *Comparison of measurement time between RD-100i and RD-200*

Note: Only the measurement time taken by RD-100*i* and RD-200 was compared. Time needed for processing specimens (homogenization of lymph node tissue, preparation of measurement samples and reagents, etc.) was not included in this evaluation.

CLINICAL PERFORMANCE

1. Setting Cut-off Values

Since the composition of the reagents for the new system has been upgraded, improving the gene amplification efficiency, the new system yields to higher values than the conventional system when using the same specimens prepared from the same lymph node. For this reason, the cut-off value was reestablished, by setting different cutoff values for the new system (cut-off points) and then comparing the result with that obtained by the conventional system for each cut-off point. The cut-off value for the new system was determined as the cut-off point for which the concordance rate with the conventional system was the maximum and for which the distance from the 100% sensitivity-specificity point to the receiver operating characteristics (ROC) curve would be the minimum.

1. Materials and Methods

1.1. Target specimen

Target specimens were derived from the lymph nodes of breast cancer patients who had undergone surgery at any of the following four institutions, and met the selection criteria listed below: The Osaka Police Hospital, The Kitakyushu Municipal Medical Center, The Sagara Hospital or The Niigata Cancer Center Hospital.

Selection criteria:
- The patient has been diagnosed with breast cancer and is indicated for surgery.
- The patient is 20 years of age or older.
- The patient has given her consent to participate in the study.

Exclusion criteria:
- The patient is suspected to have cancer other than breast cancer.
- The patient has a history of cancer other than breast cancer.
- The patient was judged to be unfit for the study by the responsible physician.
- The patient received preoperative medication other than the following: anthracycline, 5-FU, taxanes, trastuzumab, cyclophosphamide, and carboplatin.

1.2. Number of specimens

Conforming to EP24-A2 of the Clinical Laboratory Standards Institute (CLSI) and assuming the confidence interval of 95%, the significance level (α) of 5%, and the sensitivity and specificity (TPF) of 95% for the new system, the required number of specimens was calculated to be 73 or more each for positive and negative specimens according to the following formula. Therefore, a total of 288 specimens were collected to make sure to have sufficient positive and negative samples for the analysis.

$$n = \frac{\left(G(1-\alpha/2)\sqrt{TPF(1-TPF)}\right)^2}{L^2}$$

L: 1/2 of the amount outside the sensitivity's confidence interval (2.5% if 95%)
TPF: true positive fraction (sensitivity)
G(1 - α/2): (1 - α/2) percentage in the standard normal distribution (0.975 if α = 0.05)

1.3. Homogenization of lymph node tissue

Lymph node tissue specimens were homogenized with LYNORHAG to prepare the lymph node lysate. The lysate was diluted (10-fold) by using LYNORHAG to prepare "measurement samples" for both new and conventional system. In addition, for the conventional system, the lysates were further diluted (100-fold) to prepare the "diluted samples," required for the measurement.

1.4. Measurement by OSNA method

For each system, three different detectors each of which was combined with different reagent lots were used for analyzing the specimens, which were measured three times in succession. For the conventional system, the median of the nine measurement sample data and the result obtained from the corresponding diluted sample were taken as the final value. For the new system, the median of the nine measurement sample data was taken as the final value. For the (+)/(++) cut-off value, samples resulting (+)I by using the conventional system were excluded for the analysis since they can be classified neither (+) nor (++).

1.5. Data analysis

For the new system, each of the specimens was evaluated for every cut-off point. With the resolution of RD-200 detector being one second, as the rise time changes by one second the CK19 mRNA measurement changes by approximately 0.04 log copies/μL. The difference of 0.04 log copies/μL is about equal to 25 copies/μL around 250 copies/μL and 500 copies/μL around 5,000 copies/μL. Therefore, since the cut-off value of the new system should be equivalent to that of the conventional system, the (-)/(+) cut-off points were set for every 25 copies/μL and the (+)/(++) cut-off points for every 500 copies/μL.

The concordance rates were calculated as follows. (***Tables 3*** and ***4***)
Furthermore, the values corresponding to the cut-off points were plotted on a graph with the x-axis being the false positive rate (1 - specificity) and the y-axis being the sensitivity, and an ROC analysis was performed, in which distances from the upper left corner of the graph to the plotted points were used as an index.

2. Result

2.1. (-)/(+) Cut-off

The concordance rate was the greatest and the distance from the upper left corner of the graph to the ROC curve was the least at 900 and 925 copies/μL. Of the two, 900 copies/μL was chosen to be the (-)/(+) cut-off value of LYNOAMP CK19 to minimize the false negative results. (***Table 5***, ***Fig. 7***)

Table 3 *Calculation for (−)/(+) cut-off value*

		Conventional system	
		(++),(+),(+)I	(-)
New system	≥ Cut-off point	a	b
	< Cut-off point	c	d

Concordance rate: (a+d)/(a+b+c+d)×100 [%]

Table 4 *Calculation for (+)/(++) cut-off value*

		Conventional system	
		(++)	(+),(-)
New system	≥ Cut-off point	a	b
	< Cut-off point	c	d

Concordance rate: (a+d)/(a+b+c+d)×100 [%]

Table 5 *Comparison with the conventional system for each (−)/(+) cut-off point*

New system's cut-off point CK19 mRNA [copy/μL]	875	900	925	950
Concordance rate for positive ((+) and (++)) and negative (-)	99.0%	99.3%	99.3%	99.0%
Sensitivity	98.9%	98.9%	98.9%	97.8%
Specificity	99.0%	99.5%	99.5%	99.5%
ROC analysis (distance from the upper left corner of the graph)	0.0002	0.0001	0.0001	0.0005

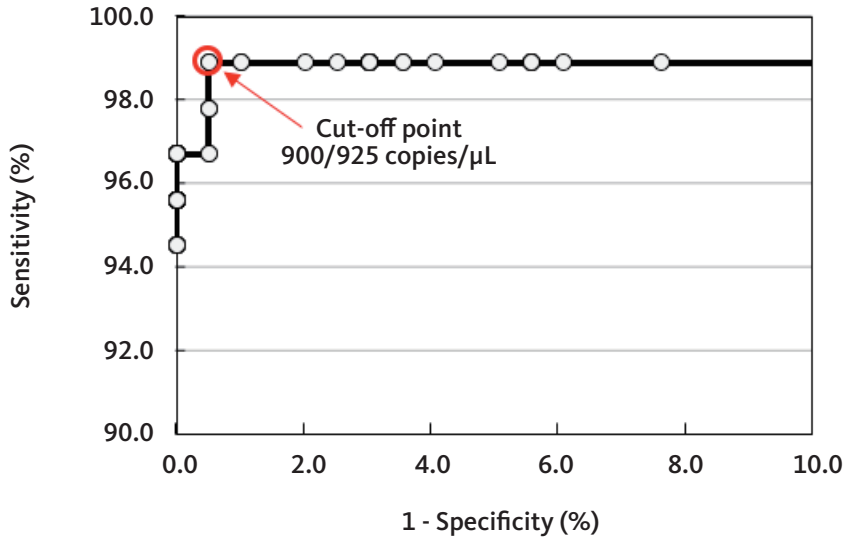

Fig. 7 *ROC curve when the new system was compared with the conventional system*

2.2. (+)/(++) Cut-off

The concordance rate was the greatest in the ranges between 14,000 and 14,500 copies/μL and between 29,500 and 30,000 copies/μL. Of the two, the 14,000-14,500 copies/μL range had the shorter distance from the upper left corner of the graph to the ROC curve. Therefore, in addition to the findings concerning the concordance rate and the ROC curve based on the sensitivity and specificity, in order to minimize the false negative results, 14,000 copies/μL was chosen to be the (+)/(++) cut-off value of LYNOAMP CK19. (***Table 6*** and ***Fig. 8***)

2. Conversion formula

In Europe, OSNA users have used the CK19 mRNA copy number values provided by the conventional system to develop additional parameters as tools for clinical decision-making in breast cancer treatment. For example, some reports propose the total tumor load (TTL) derived from the sum of CK19 mRNA copies in the sentinel lymph nodes of a breast cancer patient as a parameter for deciding whether to perform an axillary lymph node dissection. [2] Thus, in Europe the CK19 mRNA measurement by the conventional system has a broader clinical application than just the sole detection of lymph node metastasis. For this reason and to avoid any confusion by the user given the change in outcome and resulting values between systems, a conversion formula was created which converts measurements by the new system to measurements equivalent to the conventional system.

The conversion formula converting specimens' cohort was set as shown below by taking two cut-off values as reference points. Converted values from the new system are expressed

Table 6 *Comparison with the conventional system for each (+)/(++) cut-off point*

New system's cut-off point CK19 mRNA [copy/μL]	13,500	14,000	14,500	15,000	29,000	29,500	30,000	30,500
Concordance rate for strongly positive (+) and others	96.7%	97.4%	97.4%	97.1%	97.1%	97.4%	97.4%	97.1%
Sensitivity	100.0%	100.0%	100.0%	98.3%	98.1%	98.6%	98.6%	98.6%
Specificity	95.8%	96.7%	96.7%	96.7%	93.3%	93.3%	93.3%	91.7%
ROC analysis (distance from the upper left corner of the graph)	0.0018	0.0011	0.0011	0.0013	0.0048	0.0046	0.0046	0.0071

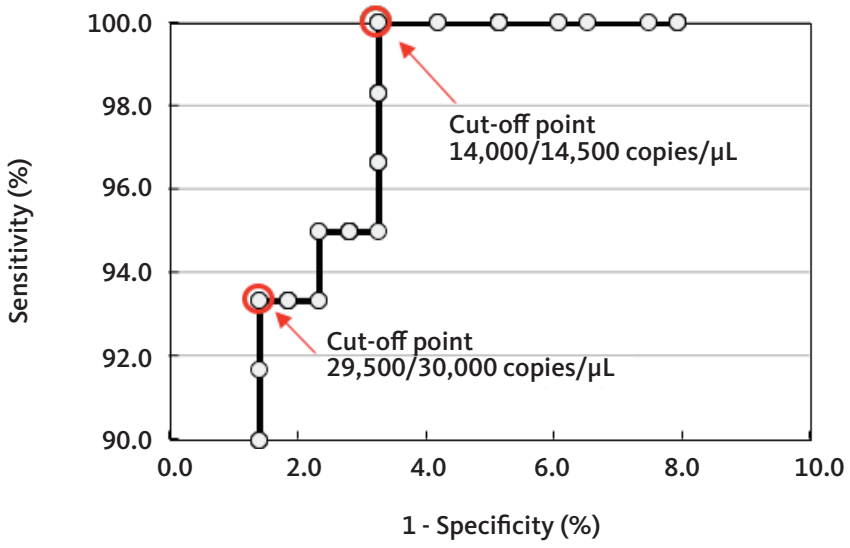

Fig. 8 *ROC curve when the new system was compared with the conventional system*

with a new unit, cCP/μL.

[Conversion formula]
y: CK19 mRNA level measured with LYNOAMP CK19
y': converted CK19 mRNA level

$$y'\ [cCP/\mu L] = 10^{\frac{Log_{10}\ y[copies/\mu L]-0.79276}{0.90345}}$$

Using the conversion formula, the (-)/(+) cut-off value of 900 copies/μL will be converted to 250 cCP/μL and the (+)/(++) cut-off value of 14,000 copies/μL to 5,000 cCP/v, conforming to the conventional systems. (***Table 7***)

To verify the validity of this conversion formula, the same set of specimens were measured by both systems, and the converted measurements from the new system were compared with the measurements from the conventional system.

1. Methods
Lysate of negative lymph node was spiked with CK19 mRNA, and ten different dilutions of the lysates were prepared (2.2×10^2, 2.7×10^2, 3.2×10^3, 3.7×10^3, 4.2×10^4, 4.7×10^4, 5.2×10^5, 5.7×10^5, 6.2×10^6 and 6.7×10^6 cCP/μL). Each diluted sample was measured successively three times, averaged and plotted on the graph, with the x-axis representing the measurements from the conventional system in log copy/μL and the y-axis the converted measurements from the new system in log cCP/μL.

2. Results
The approximated straight line drawn from the plots was y = 1.1039x -0.182 and the correlation coefficient (R^2) was 0.9975, indicating a good correlation between the converted measurements from the new system and the measurements from the conventional system. (***Fig. 9***)

Table 7 *Comparison of cut-off values*

	Conventional system	New system	
	[copy/μL]	[copy/μL]	[cCP/μL]
(+)/(++) Cut-off value	5,000 copies/μL	14,000 copies/μL	5,000 cCP/μL
(–)/(+) Cut-off value	250 copies/μL	900 copies/μL	250 cCP/μL

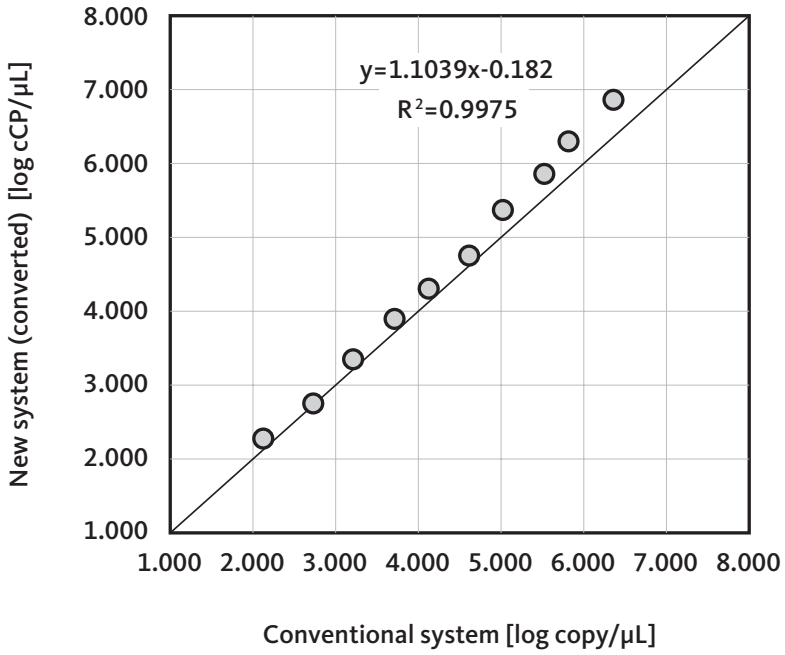

Fig. 9 *Measurement results (x-axis, measurements from conventional system in log copy/μL; y-axis, converted measurements from new system in log cCP/μL)*

3. Validation of cut-off values and omission of diluted samples

Specimens taken from the lymph node of breast cancer patients were measured both by the new system with the newly introduced cut-off values and the conventional system, and concordance rates of the positive/negative determination were evaluated.

1. Materials and Methods

1.1. Target specimens

Target samples were derived from lymph nodes of breast cancer patients who underwent surgery at any of the following three institutions, and met the selection criteria listed below: The University Hospital of Kyoto Prefectural University of Medicine, The Kindai University Hospital and The Kumamoto City Hospital.

Selection criteria:
- The patient has been diagnosed with breast cancer and is indicated for surgery.
- The patient is 20 years of age or older.
- The patient has given her consent to participate in the study.

Exclusion criteria:
- The patient is suspected to have cancer other than breast cancer.
- The patient has a history of cancer other than breast cancer.
- The patient was judged to be unfit for the study by the responsible physician.
- The patient received preoperative medication other than the following: anthracycline, 5-FU, taxanes, trastuzumab, cyclophosphamide, and carboplatin.

1.2. Number of specimens

In accordance with the standards concerning the concordance rate and the number of specimens provided in "the Number of Samples Required for Correlation Tests; Approval Standards and Test Methods [3]", the 95% confidence interval of the concordance rate was calculated to be 0.83-0.95; accordingly, the threshold value for the concordance rate was assumed to be 0.83. Assuming the expected concordance rate between the new and conventional systems to be 0.92 based on the concordance rate calculated in the preliminary studies, the number of specimens required to verify that the concordance rates do not fall below the threshold value of 0.83 with the significance level (α) and the power (1-β) being 5% and 90%, respectively, was calculated to be 146. In total 150 specimens were collected and analyzed.

1.3. Homogenization of lymph node tissue

Lymph nodes were homogenized with LYNORHAG to prepare the lymph node lysate. The lysate was diluted (10-fold) by using LYNORHAG to prepare "measurement samples" for both new and conventional system. In addition, for the conventional system, the lysates were further diluted (100-fold) to prepare the "diluted samples," required for the measurement.

1.4. Measurement by OSNA method

Each specimen was measured once. For the new system, the newly introduced cut-off values were used. (***Tables 8*** and ***9***)

Table 8 *Determination by the conventional system*

<table>
<tr><th colspan="3" rowspan="2"></th><th colspan="3">Diluted sample
CK19 mRNA
(copies/μL)</th></tr>
<tr><th>< 250</th><th>≥ 250,
< 5,000</th><th>≥ 5,000</th></tr>
<tr><td rowspan="3">Measurement sample
CK19 mRNA
(copies/μL)</td><td rowspan="2">≥ Cut-off value
(≥ 250)</td><td>≥ 5,000</td><td colspan="3">Positive (++)</td></tr>
<tr><td>≥ 250,
< 5,000</td><td colspan="2">Positive (+)</td><td>Positive (+)I</td></tr>
<tr><td>< Cut-off value
(< 250)</td><td>< 250</td><td>Negative (-)</td><td colspan="2">Positive (+)I *</td></tr>
</table>

*** In some cases, the gene amplification may be inhibited in the measurement samples, leading to copy number values <250 copies/μL for the measurement sample and of ≥250 copies/μL for the diluted sample, or between ≥250 copies/μL and <5,000 copies/μL for the measurement sample and of ≥5,000 copies/μL for the diluted sample. In this case, samples will be treated as amplification-inhibited samples (samples in which amplification has been inhibited); the result by RD-100i will be listed as (+)I and regarded as positive.**

Table 9 *Determination by the new system*

<table>
<tr><td rowspan="3">Measurement sample
CK19 mRNA
(copies/μL)</td><td rowspan="2">≥ Cut-off value
(≥ 900)</td><td>≥ 14,000</td><td>Positive (++)</td></tr>
<tr><td>≥ 900, < 14,000</td><td>Positive (+)</td></tr>
<tr><td>< Cut-off value
(< 900)</td><td>< 900</td><td>Negative (-)</td></tr>
</table>

1.5. Data analysis
The concordance rate was calculated as follows. (***Table 10***)
Concordance rate: (a+d)/(a+b+c+d) × 100 [%]

1.6. Additional measurement
If the positive/negative determination differed between the new and conventional systems, additional measurements were taken three times each by the new and conventional systems.

2. Result
The positive/negative concordance rate was 94.7%, which was considered excellent. This result verified that the cut-off values for the new system were appropriate, allowing the new system to exhibit clinical performance equivalent to that of the conventional system.

3. Discordant specimens
To closely examine the test results, the rise-times were fitted to the calibration curve to calculate measurements in the low concentration range. (***Table 11***)

Specimen No. 1, 2, 3, 4 and 5 were all negative (-) in the three additional measurements by both systems, showing a perfect concordance. Specimen No. 6 produced three times negative (-) by the conventional system but one time positive (+) and two times negative (-) by the new system. In this case, we assume that the discordance of one measurement out of three was within the extent of measurement reproducibility. (***Table 12***)

Table 10 *Calculation of the concordance rate*

			Conventional system			
			Positive			Negative
			(++)	(+)	(+)I	(-)
New system	Positive	(++)	a			b
		(+)				
	Negative	(-)	c			d

Table 11 *Results of the test*

			Conventional system			
			Positive			Negative
			(++)	(+)	(+)I	(-)
New system	Positive	(++)	26	0	2	0
		(+)	1	15	4	4
	Negative	(-)	0	3	1	94

Table 12 *Specimens of discordant determination*

Specimen No.	Conventional system					New system	
	Measurement sample		Diluted sample		Judgement	Measurement sample	
	CK19 mRNA [copy/μL]	Result	CK19 mRNA [copy/μL]	Result		CK19 mRNA [copy/μL]	Judgement
1	3.2×10^{2}	(+)	6.9×10^{-3}	(-)	(+)	1.5×10	(-)
2	2.5×10^{2}	(+)	7.8×10^{-2}	(-)	(+)	8.3×10	(-)
3	2.5×10^{2}	(+)	ND*	(-)	(+)	4.7×10	(-)
4	2.5×10	(-)	7.8×10	(-)	(-)	9.5×10^{2}	(+)
5	9.5×10	(-)	6.4×10	(-)	(-)	1.2×10^{3}	(+)
6	1.7×10^{2}	(-)	1.1	(-)	(-)	1.5×10^{3}	(+)
7	1.7×10^{2}	(-)	5.3×10^{-2}	(-)	(-)	1.7×10^{3}	(+)
8	3.9×10^{-4}	(-)	7.0×10^{2}	(+)	(+) I	1.1×10^{2}	(-)

*ND: Gene amplification did not detect within measurement time of 11 minutes.

The three additional measurements also showed that specimen No. 7 was three times negative (-) by the conventional system and three times positive (+) by the new system. Although the specimen was shown to be negative (-) by the conventional system, CK19 mRNA was detected. In addition, since this specimen was from a case where lymphatic permeation had been confirmed, lymph node metastasis was possible. Hence, it can be said that, as a certain amount of CK19 mRNA was due to be expressed, this discordance was within the extent of measurement reproducibility.

In the three additional measurements, specimen No. 8 was two times (+)I and one time (-) by the conventional system but three times (-) by the new system. In all these measurements, an amplification was observed within the measurement time. Thus, despite the values detected by the new system being lower than the cut-off value, CK19 mRNA was detected by the new system; it can be said that this discordance was within the extent of measurement reproducibility.

4. Further analysis of (+)I specimens by the conventional system

The seven specimens that were determined as (+)I by the conventional system were further analyzed. To closely examine the test results, the rise times were fitted to the calibration curve to calculate measurements in the low concentration range. (***Table 13***)

Of the seven specimens that had been determined as (+)I by the conventional system, six specimens were positive (+) also by the new system. It showed that the inhibition on gene amplification of the new system was less significant compared with the conventional system.

In the additional three measurements, specimen No. 7 was two times (+)I and one time (-) by the conventional system but three times (-) by the new system. As the specimen expressed low CK19 mRNA and showed a strong amplification inhibition, we assume that the measurement reproducibility was the cause for this discordant outcome.

It should be noted that results produced either by the new system or the conventional system should be evaluated comprehensively by physicians along with results of other relevant tests and/or clinical observations.

Table 13 *Details of (+)I specimens*

Specimen No.	Conventional system					New system	
	Measurement sample		Diluted sample		Final Result	Measurement sample	
	CK19 [copy/μL]	Result	CK19 [copy/μL]	Result		CK19 [copy/μL]	Final Result
1	3.6×10^{3}	(+)	2.5×10^{5}	(++)	(+) I	1.4×10^{5}	(++)
2	2.3×10^{3}	(+)	7.8×10^{3}	(++)	(+) I	4.2×10^{4}	(++)
3	2.3×10^{3}	(+)	3.9×10^{4}	(++)	(+) I	7.6×10^{3}	(+)
4	1.5×10^{2}	(-)	1.0×10^{3}	(+)	(+) I	3.4×10^{3}	(+)
5	2.2×10^{2}	(-)	3.2×10^{2}	(+)	(+) I	2.2×10^{3}	(+)
6	1.7×10^{2}	(-)	2.5×10^{2}	(+)	(+) I	1.5×10^{3}	(+)
7	3.9×10^{-4}	(-)	7.0×10^{2}	(+)	(+) I	1.1×10^{2}	(-)

ANALYTICAL PERFORMANCE

Introduction of any diagnostic system to a clinical setting requires evaluation of analytical performance to determine and maintain the high level of diagnostic precision. Therefore, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guideline issued by the National Committee for Clinical Laboratory Standards (NCCLS), tissue specimens were measured by the new system to evaluate the analytical performance. (***Table 14***)

1. Single-site Precision

Reproducibility was analyzed at the same institution, on the same detector, and by the same operator.

1. Methods
1.1. Target specimen
Lysates of negative lymph node were spiked with CK19 mRNA at five different concentrations (1.6×10^2, 2.5×10^2, 5.2×10^3, 2.8×10^4 and 2.4×10^6 cCP/μL).

1.2. Measurement
Each specimen was measured four times (two replicates/run and two runs) per day for 20 days (ten days/single lot LYNOAMP CK19).

1.3. Data analysis
The single-site precision of each sample was calculated according to the CLSI guideline EP05-A3.

2. Results
The single-site precision of each specimen was calculated as shown in ***Table 15***.

Table 14 *Analytical performance when specimens were measured in log cCP/μL*

Item	Result (analyzed in log cCP/μL)
Single-site Precision	Specimen A (1.6×10^2 cCP/μL): CV 8.2%
	Specimen B (2.5×10^2 cCP/μL): CV 4.9%
	Specimen C (5.2×10^3 cCP/μL): CV 2.5%
	Specimen D (2.8×10^4 cCP/μL): CV 1.6%
	Specimen E (2.4×10^6 cCP/μL): CV 1.0%
Multi-site Precision (Reproducibility)	Specimen A (1.6×10^2 cCP/μL): CV 9.2%
	Specimen B (2.5×10^2 cCP/μL): CV 7.9%
	Specimen C (5.2×10^3 cCP/μL): CV 3.3%
	Specimen D (2.8×10^4 cCP/μL): CV 2.3%
	Specimen E (2.4×10^6 cCP/μL): CV 1.2%
Bias	Greatest value for 3 reagent lots: 0.309 log cCP/μL
Accuracy (total analytical error)	Greatest value for 3 reagent lots: 0.889 log cCP/μL
Limit of blank (LoB)	13 cCP/μL (63 copies/μL)
Limit of detection (LoD)	56 cCP/μL (240 copies/μL)
Limit of quantification (LoQ)	160 cCP/μL (600 copies/μL)
Measuring range	Between 2.200 log cCP/μL and 8.200 log cCP/μL (Between 160 cCP/μL and 1.6×10^8 cCP/μL)

Table 15 *Single-site precision*

Target specimen	Theoretical value		CV (%)	Single-site precision (mean ± SD)	
	(cCP/μL)	(log cCP/μL)		(cCP/μL)	(log cCP/μL)
A	1.6×10^2	2.200	8.2	1.1×10^2 - 2.7×10^2	2.058 - 2.425
B	2.5×10^2	2.392	4.9	2.4×10^2 - 4.2×10^2	2.382 - 2.628
C	5.2×10^3	3.712	2.5	4.9×10^3 - 7.4×10^3	3.686 - 3.872
D	2.8×10^4	4.454	1.6	2.6×10^4 - 3.7×10^4	4.422 - 4.567
E	2.4×10^6	6.382	1.0	4.7×10^6 - 6.4×10^6	6.674 - 6.809

2. Multi-site Precision (Reproducibility)

The reproducibility was analyzed when measurements were taken at different institutions, on different detectors, and by different operators.

1. Methods
1.1. Target specimen
Lysates of negative lymph nodes were spiked with CK19 mRNA at five different concentrations (1.6×10^2, 2.5×10^2, 5.2×10^3, 2.8×10^4 and 2.4×10^6 cCP/μL).

1.2. Measurement
Each sample was measured at three different sites by three different operators using three different instruments. In total, each sample was measured 54 times per site (three times per run, three runs per day, for two days per reagent lot, with three different reagent lots).

1.3. Data analysis
The multi-site precision of each specimen was calculated according to the CLSI guideline EP05-A3.

2. Results
The multi-site precision of each specimen was calculated as shown in ***Table 16***.

3. Bias

For the concentration range of the measurement, errors of the expected values of the measurements from the theoretical values were analyzed.

1. Methods
1.1. Target specimen
Lysates of ten negative lymph nodes were spiked with CK19 mRNA in 12 different concentrations (160, 500, 1.6×10^3, 5.0×10^3, 1.6×10^4, 5.0×10^4, 1.6×10^5, 5.0×10^5, 1.6×10^6, 5.0×10^6, 1.6×10^7, and 5.0×10^7 cCP/μL).

1.2. Measurement
Each of the samples was measured with three different lots of LYNOAMP CK19 E across four days. Each sample was measured once per reagent lot, and a total 120 data points of each reagent lot were analyzed separately.

1.3. Data analysis
The bias between target and measured concentration was calculated for each reagent lot according to the CLSI guideline EP09-A3.

2. Results
Bias was 0.309 log cCP/μL (highest value through three reagent lots).

4. Accuracy (Total Analytical Error)

From the bias and reproducibility, possible errors in the measurements from the theoretical values were analyzed.

1. Methods
1.1. Target specimen
Lysate of ten negative lymph node were spiked with CK19 mRNA in 12 different concentrations (160, 500, 1.6×10^3, 5.0×10^3, 1.6×10^4, 5.0×10^4, 1.6×10^5, 5.0×10^5, 1.6×10^6, 5.0×10^6, 1.6×10^7, and 5.0×10^7 cCP/μL) .

1.2. Measurement
Each of the samples was measured with three different lots of LYNOAMP CK19 across four days. Each sample was measured once per reagent lot, and a total 120 data points for each reagent lot were analyzed separately.

Table 16 *Multi-site precision*

Target specimen	Theoretical value		CV (%)	Multi-site precision (mean ± SD)	
	(cCP/μL)	(log cCP/μL)		(cCP/μL)	(log cCP/μL)
A	1.6×10^2	2.200	9.2	1.6×10^2 - 4.6×10^2	2.213 - 2.659
B	2.5×10^2	2.392	7.9	2.7×10^2 - 7.0×10^2	2.425 - 2.842
C	5.2×10^3	3.712	3.3	4.7×10^3 - 8.4×10^3	3.676 - 3.925
D	2.8×10^4	4.454	2.3	3.0×10^4 - 4.8×10^4	4.472 - 4.685
E	2.4×10^6	6.382	1.2	4.1×10^6 - 5.8×10^6	6.612 - 6.767

1.3. Data analysis
Total analytical error of each reagent lot was calculated according to the CLSI guideline EP21-A.

2. Results
Total analytical error (TAE) was 0.889 log cCP/μL (highest value through three reagent lots).

5. Limit of blank (LoB)

1. Methods
1.1. Target specimen
Lysates of four negative lymph nodes

1.2. Measurement
Lysates were measured with three different lots of LYNOAMP CK19. Each sample was measured 15 times (five times per day, for three days) per reagent lot, and a total 60 data points of each reagent lot were analyzed separately.

1.3. Data analysis
LoB of each reagent lot was calculated according to the classical approach in CLSI guideline EP17-A2, and the highest LoB value through three different lots was taken as LoB of LYNOAMP CK19 E.

2. Results
The LoB was calculated to be 13 cCP/μL (63 copies/μL).

6. Limit of detection (LoD)

1. Methods
1.1. Target specimen
Lysates of negative lymph node were spiked with CK19 mRNA at five different concentrations (10, 16, 25, 40 and 63 cCP/μL).

1.2. Measurement
Each specimen was measured with three different lots of LYNOAMP CK19. In total, each sample was measured 12 times (four times per day, for three days) per reagent lot, and a total 60 data points of each reagent lot were analyzed separately.

1.3. Data analysis
LoD of each reagent lot was calculated according to the classical approach in CLSI guideline EP17-A2, and the highest LoD value through three different lots was taken as LoD of LYNOAMP CK19.

2. Results
The LoD was calculated to be 56 cCP/μL (240 copies/μL).

7. Limit of quantification (LoQ)

1. Methods
1.1. Target specimen
Lysates of negative lymph nodes were spiked with CK19 mRNA at six different concentrations (20, 40, 79, 160, 320 and 630 cCP/μL).

1.2. Measurement
Each sample was measured nine times (three times per day, for three days) per reagent lot and each reagent lot was analyzed separately.

1.3. Data analysis
The lowest value fulfilling the criteria of total analytical error and precision of measured concentration was determined as LoQ of each reagent lot, and the highest LoQ value through the three lots was taken as LoQ of LYNOAMP CK19.

2. Results
The LoQ was calculated to be 160 cCP/μL (600 copies/μL).

8. Measuring range

1. Methods
1.1. Target specimen
Lysates of negative lymph nodes were spiked with CK19 mRNA at nine different concentrations (160, 500, 1.6×10^3, 5.0×10^3, 1.6×10^4, 5.0×10^4, 1.6×10^6, 1.6×10^7 and 1.6×10^8 cCP/μL).

1.2. Measurement
Each of the specimens was measured three times successively using three reagent lots.

1.3. Data analysis
The measurements were analyzed for each reagent lot in accordance with EP06-A, a CLSI guideline, to confirm linearity in the measuring range in each reagent lot according to the CLSI guideline EP06-A..

2. Results
The measuring range of LYNOAMP CK19 was between 2.200 and 8.200 log cCP/μL (between 160 and 1.6×10^8 cCP/μL).

CONCLUSION

Sysmex Corporation has developed a new system (the automated Gene Amplification Detector RD-200 and LYNOAMP CK19 reagent) using the One-Step Nucleic Acid Amplification (OSNA) method. Improvements from the conventional system (the automated Gene Amplification Detector RD-100*i* and LYNOAMP BC reagent) were summarized in ***Table 17***.

In comparison to the conventional system, the new system is easier to use, provides faster results, allows the increased simultaneous analysis of up to 14 samples, and offers a multi-application platform that allows the analysis of lymph nodes in different cancer types. In addition, the newly established result conversion formula with "cCP/μL" unit facilitates straightforward data comparison between the two OSNA systems for easy adoption by customers.

Moreover, the new system uses the newly introduced cut-off values and does not require diluted samples.

The clinical performance of the new system has been compared in a clinical trial to that of the conventional system. The findings clearly demonstrate that the clinical performance of both systems is equivalent.

The analytical performance of the new system in measuring samples derived from human lymph nodes has also been evaluated. The results of the analytical test which was done in accordance with the CLSI guidelines are expected to become reference data for quality control.

Taken together, the new OSNA system developed by Sysmex Corporation carries several advantages when compared to the previous generation and has been designed to fulfill the user's needs as an easy, fast and accurate tool for the diagnosis of lymph node metastasis. Moreover, the multi-application feature offers the possibility to expand the number of cancer types that can be analyzed by using the OSNA method, further confirming the great potential of this innovative technology.

Table 17 *Comparison between the new and conventional systems*

	Conventional system RD-100*i* and LYNOAMP BC	New system RD-200 and LYNOAMP CK19
	 	 
Measurement time (from loading the detector to receiving result)	24.5 min per measurement in the case of two-sample measurement	17 min per measurement in the case of two-sample measurement
Analytical performance	Qualitative (−)/(+) cut-off value, 250 copies/μL; (+)/(++) cut-off value, 5,000 copies/μL	Qualitative (−)/(+) cut-off value, 900 copies/μL (250 cCP/μL) (+)/(++) cut-off value, 14,000 copies/μL (5,000 cCP/μL)
Target sample	Measurement sample and diluted sample	Measurement sample (diluted sample not required)
Number of samples that can be measured	Maximum of 4 lymph nodes per measurement	Maximum of 14 lymph nodes per measurement
Specifications of reagents	240 tests/kit Expiration period after opening, 1 month	120 tests/kit Expiration period after opening, 2 months
Usability features	—	Remaining reagent management by QR code; space-saving design with built-in control panel; enhanced internal precision control function; reagent container of lid-body integrated design

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