Basic Evaluation of Lynoamp BC Used for Intra-Operative Detection of Lymph Node Metastasis in Breast Cancer Patients

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In 2008, OSNA (One Step Nucleic acid Amplification) was approved by the Ministry of Health in Japan. Following the approval, OSNA has been applied as a routine diagnostic tool for rapid detection of metastases in sentinel lymph node samples from breast cancer patients.

We performed the basic evaluation of Lynoamp BC on the basis of the following criteria.

1) Reproducibility, 2) Linearity, 3) Inter-vial variability test, 4) Positive and negative accuracy, 5) Long-term stability, 6) Interference materials,

As a result, it turned out that the result of the basic evaluation of Lynoamp BC was excellent. We expect that OSNA can contribute to standardizing breast cancer treatment by minimizing the known variability in the methodology and accuracy of conventional SLN diagnosis.

Key Words OSNA, RD-100*i*, Lynoamp BC, Cytokeratin 19 (CK19)

INTRODUCTION

One Step Nucleic acid Amplification (OSNA) is an assay that can amplify and detect, without purification, the cytokeratin 19 (CK19) mRNA of cancer cell origin in breast cancer-affected lymph nodes, by the combined use of a solubilizing reagent Lynorhag and a gene amplifying reagent Lynoamp BC. The steps, from dispensing of Lynoamp BC and samples to gene amplification and detection by the OSNA assay, have been automated in a dedicated detector (RD-100*i*, Sysmex Corporation). This assay system has the easy of use and speed suitable for use in intraoperative rapid testing of metastasis.

A large-scale evaluation of the clinical performance of the OSNA assay undertaken at 7 sites during 2005-2006 showed high specificity (97.1%) and high concordance (92.9%) with the results of pathologic examination, and the assay is already in clinical use at the point of care both in Japan and abroad.

In the present study, we evaluated the basic performance of Lynoamp BC, the special reagent used in the OSNA assay.

CHARACTERISTICS OF LYNOAMP BC

Lynoamp BC is a gene amplification reagent meant for exclusive use with the OSNA assay for detecting CK19 mRNA in the dissected regional lymph nodes [sentinel lymph nodes (SLN) in particular] of breast cancer patients (for assisting the diagnosis of lymph node metastasis of breast cancer). Analyses of patient samples using this reagent have been covered for reimbursement under the National Health Insurance scheme from November 2008.

240 assays (including appropriate assay control samples and the assays for preparing the calibration curve) can be carried out with one assay kit, which is meant to be stored at -20 (±5)°C, where it is stable for 12 months if the seal is not opened (one month after the seal is opened) (***Fig. 1***).

For this assay, lymph nodes (SLN) of breast cancer patients are solubilized with Lynorhag. This solubilized lymph node preparation is then mixed with a CK19 primer solution and the enzyme solution, and allowed to

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react at a constant temperature of 65°C. In the first step of the reaction, cDNA is synthesized by the reverse transcriptase using the CK19 mRNA in the solubilized lymph node preparation as the template. The amplification of this cDNA advances through reverse transcription loop mediated isothermal amplification (RT-LAMP) by the action of DNA synthase. This DNA amplification is detected by measuring the time taken for the turbidity caused by magnesium pyrophosphate, a white precipitate that is a byproduct of the reaction, to cross a threshold value. As there is a linear relationship between the CK19 mRNA concentration and the "Rise time", i.e., the time elapsed from the start of the reaction to the point where the turbidity crosses the threshold, the concentration of the CK19 mRNA in the assay sample can be determined using a calibration curve prepared beforehand with known concentrations of CK19 mRNA. The assessment of breast cancer metastasis within the lymph node is made by comparing the measured CK19 mRNA concentration with a predetermined cutoff value (***Fig. 2***).

Cytokeratin 19 mRNA kit - Lynoamp BC

INTENDED PURPOSE	For the detection of CK19 mRNA in surgically removed regional lymph node in breast and colorectal cancer with the Gene Amplification Detector RD-100*i*.
Storage	Storage: -20 (±5)°C
Validity after seal is opened	1 month
Packaging unit	CK19 primer solution: 720 µL × 8 Enzyme solution: 450 µL × 2 CK19 positive control: 110 µL × 4 CK19 negative control: 110 µL × 4 Level 1 calibrator: 110 µL × 4 Level 2 calibrator: 110 µL × 4 Level 3 calibrator: 110 µL × 4

Fig. 1 *Composition of the Lynoamp BC kit*

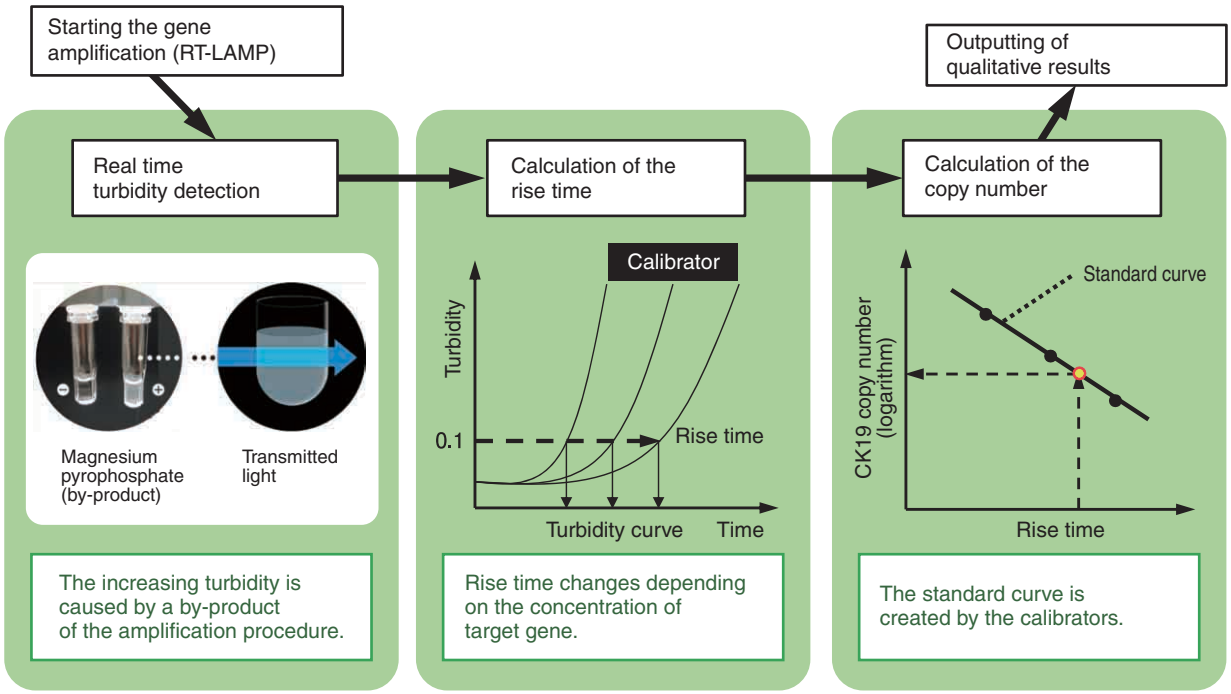


Fig. 2 *Gene amplification and detection using RD-100i - Turbidity detection method*

PARAMETERS ANALYZED AND METHODS USED

The basic performance of Lynoamp BC with regard to the parameters described below was evaluated using 3 different lots of Lynoamp BC (ZS8004, ZS8005, and ZS9002).

1. Within-run reproducibility

Three calibrators of different concentrations (CK19-C1, CK19-C2, and CK19-C3) were each measured 10 times consecutively and the mean value, standard deviation, and the coefficient of variation (CV (%)) of the Rise time were calculated.

2. Linearity

A 6-step dilution series with a defined dilution ratio was prepared within the CK19 mRNA concentration range 2.5×10^2 - 2.5×10^7 copies/μL, and 3 replicate measurements were made at each concentration. The mean value of the measured concentration was obtained for each sample and the log-log linear regression equation (Y = a+bX) between the measured and theoretical concentrations of the dilution series was calculated.

3. Inter-vial variability test

Four vials of calibrators from the same lot were each measured once and the mean Rise time ($Xbar_1$) and standard deviation (S_1) were calculated.
Next, 4 replicate measurements were made on the sample in 1 of the 4 vials and mean Rise time ($Xbar_2$) and standard deviation (S_2) were calculated. The inter-vial CV (%) was calculated using the equation given below to eliminate the effect of within-run reproducibility-related factors.

$S_{\text{inter-vial}} = \sqrt{S^2_1 - S^2_2}$
$CV_{\text{inter-vial}}(\%) = S_{\text{inter-vial}} / Xbar_1 \times 100$
When $S_1 < S_2$, $CV_{\text{inter-vial}}(\%) = 0$

4. Positive and negative accuracy

The agreement in assessment was studied using CK19 mRNA-containing reference samples (2 positive reagents: CK19-PC and CK19-C1) and epithelium-derived cultured cell lines (3 cell lines: SKBr3, KATO III, LC-2/ad, each at 1×10^5 cells/reaction) as positive samples, and reference samples containing no CK19-mRNA (CK19-NC, yeast RNA, RD reagent C, Lynorhag, and physiological saline) as negative samples.

5. Long-term stability

The performance parameters 1 to 4 described above were evaluated in the same manner except for using reagent vials stored unopened up to the end of their validity period.

6. Effect of interfering substances

Lymph nodes (SLN) of breast cancer patients, which are the targets of the OSNA assay, may contain substances that could possibly affect the gene amplification reaction. Such substances include the patient's blood, and dyes and RI reagents used for identifying SLN. We examined the effect of the presence of such interfering substances on the amplification reaction under the conditions described below.

1) Blood
Blood was added to a final concentration of 0.0 - 6.0% to CK19 mRNA solutions of 3 different concentrations (high, low and zero) and changes in the results of analysis before and after the addition were examined (n = 2).

2) Dyes - Diagnogreen and indigocarmine
Diagnogreen (Daiichi Sankyo) to a final concentration of 0.0 - 0.125% or indigocarmine (Daiichi Sankyo) to a final concentration of 0.0 - 0.02% was added to CK19 mRNA solutions of 3 different concentrations (high, low and zero) and changes in the results of analysis before and after the addition were examined (n = 2).

3) RI reagents - Tin colloid and phytic acid
Tin colloid (kit for the preparation of Tc-99m tin colloid injection, Nihon Medi-Physics) or phytic acid (Techne Phytate Kit, Fujifilm RI Pharma) was added to a concentration of 0.0 - 1.0 (V/V %) to a CK19 mRNA solution of final concentration 1.0×10^4 copies/μL. The change in Rise time before and after the addition was examined (n = 5).

RESULTS

1. Within-run reproducibility

The CV was not more than 4% for low concentration reagent (CK19-C1) of all the lots. Furthermore, at medium concentration (CK19-C2) and high concentration (CK19-C3) the CV was not more than 2%, which represented good reproducibility (***Table 1***).

2. Linearity

Good linearity was seen in the range 2.5×10^2 - 2.5×10^7 copies/μL, with correlation coefficients of 0.900 or higher (***Fig. 3***).

Table 1 *Simultaneous reproducibility*

Lot No.	ZS8004			ZS8005			ZS9002		
Number of times	CK19-C1 (2.5×10^3 cp)	CK19-C2 (2.5×10^5 cp)	CK19-C3 (2.5×10^7 cp)	CK19-C1 (2.5×10^3 cp)	CK19-C2 (2.5×10^5 cp)	CK19-C3 (2.5×10^7 cp)	CK19-C1 (2.5×10^3 cp)	CK19-C2 (2.5×10^5 cp)	CK19-C3 (2.5×10^7 cp)
1	10.9	9.7	8.7	11.3	9.7	8.7	10.5	9.5	8.6
2	11.2	9.7	8.7	11.0	9.7	8.8	10.8	9.5	8.6
3	10.5	9.6	8.7	11.0	9.7	8.7	10.6	9.5	8.5
4	11.0	9.6	8.7	10.6	9.7	8.7	10.7	9.6	8.6
5	10.3	9.6	8.7	10.4	9.7	8.7	10.4	9.5	8.5
6	11.5	9.7	8.7	11.0	9.7	8.7	10.5	9.5	8.5
7	11.0	9.6	8.7	11.1	9.7	8.7	10.8	9.5	8.5
8	11.1	9.6	8.7	10.7	9.7	8.7	11.0	9.5	8.6
9	11.3	9.6	8.7	10.7	9.7	8.7	10.4	9.5	8.5
10	10.9	9.7	8.7	10.6	9.6	8.7	10.6	9.5	8.6
AVE	11.0	9.6	8.7	10.8	9.7	8.7	10.6	9.5	8.6
SD	0.4	0.1	0.0	0.3	0.0	0.0	0.2	0.0	0.1
CV	3.6%	1.0%	0.0%	2.8%	0.0%	0.0%	1.9%	0.0%	1.2%

* cp = copies/μL

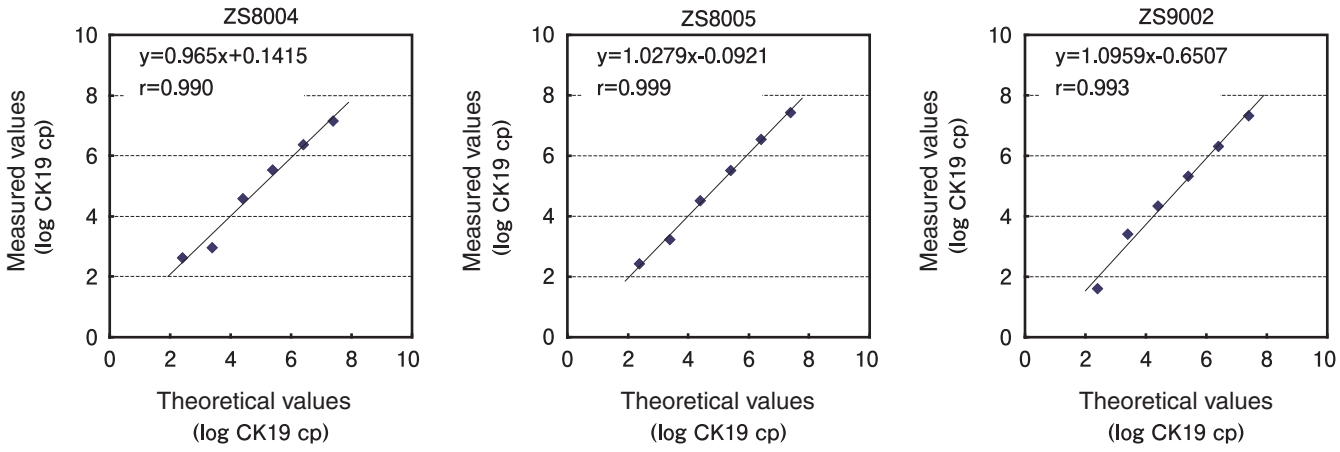


Fig. 3 *Linearity*

A 6-step dilution series with a constant dilution ratio was prepared within the CK19 mRNA range of 2.5×10^2 - 2.5×10^7 copies/μL (n = 3 for each concentration).

** cp = copies/μL*

3. Inter-vial variation

The inter-vial CV was not more than 3%, which confirmed that there was no difference between vials of the same kit (***Table 2***).

4. Positive and negative accuracy

All the 5 positive reference samples were assayed positive and all the negative samples, which did not contain any CK19 mRNA, were assayed negative (***Table 3***).

Table 2 *Inter-vial differences*

	ZS8004			ZS8005			ZS9002		
Vial	CK19-C1	CK19-C2	CK19-C3	CK19-C1	CK19-C2	CK19-C3	CK19-C1	CK19-C2	CK19-C3
1	10.6	9.8	8.7	10.6	9.7	8.7	11.0	9.5	8.5
2	10.9	9.7	8.8	10.9	9.8	8.7	10.8	9.5	8.5
3	10.8	9.7	8.7	10.8	9.7	8.7	10.7	9.5	8.5
4-1	11.2	9.8	8.8	10.8	9.7	8.7	10.5	9.5	8.5
4-2	10.8	9.7	8.8	10.9	9.7	8.7	10.9	9.5	8.5
4-3	10.9	9.7	8.8	11.1	9.7	8.7	10.5	9.5	8.5
4-4	11.4	9.7	8.7	10.9	9.7	8.7	10.8	9.5	8.6
S1	0.3	0.0	0.1	0.1	0.0	0.0	0.2	0.0	0.0
S2	0.3	0.0	0.1	0.1	0.0	0.0	0.2	0.0	0.0
X1	10.9	9.8	8.8	10.8	9.7	8.7	10.8	9.5	8.5
CV (vial)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Table 3 *Positive and negative accuracy*

Accuracy in positive assessment

Sample		ZS8004	ZS8005	ZS9002
Positive reagent	PC	(++)	(++)	(+)
	C1-1	(++)	(+)	(+)
Cell lines $1x10^5$ (cell/reaction)	SKBr3	(++)	(++)	(++)
	KATOⅢ	(++)	(++)	(++)
	LC2/ad	(++)	(+)	(++)

Accuracy in negative assessment

Sample	ZS8004	ZS8005	ZS9002
NC	(−)	(−)	(−)
YeastRNA	(−)	(−)	(−)
RD reagentC	(−)	(−)	(−)
LYNORHAG	(−)	(−)	(−)
Physiological saline	(−)	(−)	(−)

Determined using a calibration curve obtained while evaluating within-run reproducibility (first round)

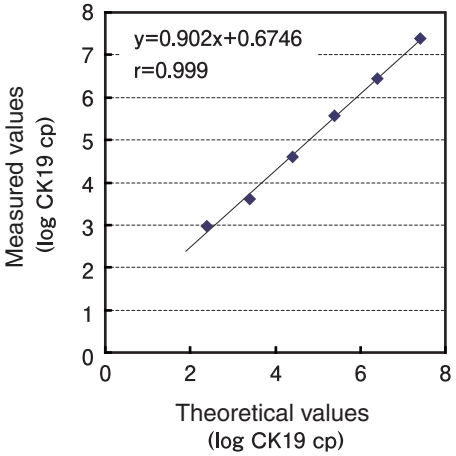
5. Long-term stability

Reagents stored up to the end of their period of validity were found to have the same level of performance as those within their period of validity. The results thus confirmed that as long as the reagent was stored appropriately, i.e., at -20 (±5)°C, there was no problem with regard to long-term stability of Lynoamp BC (***Fig. 4***).

Simultaneous reproducibility

Number of times	CK19-C1 (2.5×10^3 cp)	CK19-C2 (2.5×10^5 cp)	CK19-C3 (2.5×10^7 cp)
1	10.8	9.5	8.5
2	11.3	9.5	8.5
3	11.0	9.4	8.4
4	10.4	9.5	8.5
5	10.4	9.4	8.5
6	11.0	9.4	8.5
7	10.8	9.5	8.5
8	11.0	9.5	8.5
9	10.5	9.5	8.4
10	10.5	9.4	8.5
AVE	10.8	9.5	8.5
SD	0.3	0.1	0.0
CV (%)	2.8%	1.1%	0.0%

Linearity



Inter-vial differences

Vial	CK19-C1	CK19-C2	CK19-C3
1	10.8	9.5	8.5
2	10.5	9.5	8.5
3	11.2	9.4	8.5
4-1	11.3	9.5	8.5
4-2	11.2	9.4	8.4
4-3	11.0	9.5	8.5
4-4	10.6	9.5	8.5
S1	0.4	0.1	0.0
S2	0.3	0.1	0.1
X1	11.0	9.5	8.5
CV(vial)	2.4%	0.0%	0.0%

Positive accuracy

Sample		ZS8003
Positive reagent	PC	(+)
	C1-1	(+)
Cell lines $1x10^5$ (cell/reaction)	SKBr3	(++)
	KATOⅢ	(++)
	LC2/ad	(++)

Negative accuracy

Sample	ZS8003
NC	(−)
YeastRNA	(−)
RD reagentC	(−)
LYNORHAG	(−)
Physiological saline	(−)

Fig. 4 *Long-term stability*

Performance was studied in the same way as earlier, except for using reagents kept sealed until the end of the validity period.

** cp = copies/μL*

6. Effect of interfering substances (*Fig. 5*)

1) Blood

Addition of blood to CK19 solutions of different concentrations showed that the presence of blood up to 6.0% concentration did not affect the assay results.

2) Dyes - Diagnogreen and indigocarmine

Addition of Diagnogreen up to 0.125% or indigocarmine up to 0.020% to CK19 solutions of different concentrations did not affect the assay results.

3) RI reagents - Tin colloid and phytic acid

The solution to which no tin colloid had been added (control) had a rise-up time of 10.2 ± 0.1 minutes (mean ± 2 SD). Similarly, the solution containing no phytic acid (control) had a rise-up time of 10.3 ± 0.2 minutes. Addition of tin colloid or phytic acid up to 1.00 V/V% to the sample did not affect the rise-up time.

Blood

Blood concentration in the assay sample (%)	High concentration CK19 mRNA solution		Low concentration CK19 mRNA solution		CK19 mRNA negative solution	
	Rise-up time (min)	Assess-ment	Rise-up time (min)	Assess-ment	Rise-up time (min)	Assess-ment
0.0	8.6	(++)	10.4	(+)	ND	(−)
0.1	8.7	(++)	10.4	(+)	ND	(−)
0.3	8.7	(++)	10.2	(+)	ND	(−)
0.5	8.8	(++)	10.3	(+)	ND	(−)
1.0	8.8	(++)	10.5	(+)	ND	(−)
2.0	8.7	(++)	10.4	(+)	ND	(−)
4.0	8.6	(++)	10.3	(+)	ND	(−)
6.0	8.7	(++)	10.5	(+)	ND	(−)
AVE	8.7		10.4			
SD	0.1		0.1			
CV (%)	1.1%		1.0%			

Dye (Diagnogreen)

Dye concentration in the assay sample (%)	High concentration CK19 mRNA solution		Low concentration CK19 mRNA solution		CK19 mRNA negative solution	
	Rise-up time (min)	Assess-ment	Rise-up time (min)	Assess-ment	Rise-up time (min)	Assess-ment
0.000	9.1	(++)	10.8	(+)	ND	(−)
0.003	9.1	(++)	10.7	(+)	ND	(−)
0.050	9.1	(++)	10.7	(+)	ND	(−)
0.125	9.1	(++)	10.8	(+)	ND	(−)
AVE	9.1		10.7			
SD	0.0		0.1			
CV (%)	0.0%		0.9%			

Diagnogreen (Daiichi Sankyo)

Dye (indigocarmine)

Dye concentration in the assay sample (%)	High concentration CK19 mRNA solution		Low concentration CK19 mRNA solution		CK19 mRNA negative solution	
	Rise-up time (min)	Assess-ment	Rise-up time (min)	Assess-ment	Rise-up time (min)	Assess-ment
0.000	8.3	(++)	9.9	(+)	ND	(−)
0.004	8.3	(++)	9.8	(+)	ND	(−)
0.008	8.3	(++)	9.7	(+)	ND	(−)
0.016	8.3	(++)	9.9	(+)	ND	(−)
0.020	8.3	(++)	9.8	(+)	ND	(−)
AVE	8.3		9.8			
SD	0.0		0.1			
CV (%)	0.0%		1.0%			

Indigocarmine (Daiichi Sankyo)

RI reagents

Concentration in the assay sample (V/V%)	Tin colloid solution	Phytic acid solution
	Rise-up time (min), mean ± 2SD (n=5)	
Control (0)	10.2±0.1	10.3±0.2
0.01	10.3±0.1	10.2±0.1
0.10	10.3±0.2	10.3±0.3
1.00	10.2±0.2	10.2±0.2

Kit for the preparation of Tc-99m tin colloid injection (Nihon Medi-Physics)
Techne Phytate Kit (Fujifilm RI Pharma)

Fig. 5 *Interfering substances*

DISCUSSION

In the present study, we evaluated the analytical performance of Lynoamp BC. The accuracy of the assay system is important for correctly carrying out genetic tests. Usually, with gene amplification methods like PCR, various proteins and blood present in the sample affect the amplification reaction, and this makes it essential to purify the nucleic acid. However, increasing the number of steps in a process increases the risk of variability within that process (poor extraction, poor purificaion, degradation with time, etc. of the nucleic acid) that could potentially affect the analysis data, which in turn could affect the analysis results. In the OSNA assay, which uses Lynoamp BC, the majority of processes have been automated in the dedicated detector (RD-100*i*), and no cumbersome operations such as extensive and often manual RNA purification techniques are needed. At the same time, the assay is not affected by the presence of blood derived from the specimen, or interfering substances used during the surgery. Therefore, we can say that it is an assay method where the effects of nucleic acid degradation and nucleic acid extraction error can be minimized. Furthermore, in addition to internal quality control of the data done for every batch with CK19 positive and negative controls, external quality control can be implemented by connecting to the Sysmex Network Communication Systems (SNCS) where the data can be compared with that from other medical institutions. We believe that all these features make it possible for any medical facility to perform lymph node metastasis testing of uniformly high quality.

CONCLUSION

Testing of breast cancer metastasis in lymph nodes with Lynoamp BC is an accurate, reproducible, consistent, quantitative, cost effective and easy to use assay. It is hoped that intraoperative metastasis testing would become standardized as the clinical use of the OSNA assay spreads further in the future.

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