Rapid diagnosis of acute promyelocytic leukemia with the *PML-RARA* fusion gene using a combination of droplet-reverse transcription-polymerase chain reaction and instant-quality fluorescence in situ hybridization

Shohei Shigeto,[a] Kazuyuki Matsuda,[a]# Akemi Yamaguchi,[b] Akane Sueki ,[a] Masayuki Uehara,[b] Mitsutoshi Sugano,[a] Takeshi Uehara,[a] Takayuki Honda[a]

Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan[a]; Core Technology Development Center, Seiko Epson Corporation, Suwa, Japan[b]

Correspondence: Kazuyuki Matsuda, PhD, Department of Laboratory Medicine, Shinshu University Hospital, 3-1-1, Asahi, Matsumoto, 390-8621, Japan.

TEL: +81-263-37-2802; FAX: +81-263-34-5316; E-mail: kmatsuda@shinshu-u.ac.jp

ABSTRACT

Background: Acute promyelocytic leukemia (APL) with the *PML-RARA* fusion gene can be effectively cured using molecular-targeted therapies, which require both detection and quantification of the *PML-RARA* fusion gene. Here, we developed a rapid assay for identifying and measuring the *PML-RARA* fusion gene in patients with APL using droplet-reverse transcription-polymerase chain reaction (droplet-RT-PCR) and instant quality-fluorescence in situ hybridization (IQ-FISH).

Methods: RNA for droplet-RT-PCR and fixed-cell suspensions for IQ-FISH were prepared from five patients with APL and three controls. We evaluated the amplification efficiency and reaction time with droplet-RT-PCR and signal clarity and hybridization time with IQ-FISH.

Results: The reaction using droplet-RT-PCR was completed in 26 min. The *PML-RARA* fusion gene was detected in all samples from the five patients. IQ-FISH yielded clear signals after 1 h of hybridization. There were no significant differences in signal clarity or positive signal ratios between IQ-FISH and conventional FISH.

Conclusions: Simultaneous droplet-RT-PCR and IQ-FISH, in addition to morphological examination of blood smears, can be used to diagnose patients as having APL within 4 h based on molecular/cytogenetic results. Rapid diagnosis can allow effective therapies to be started promptly.

Abbreviations: APL, acute promyelocytic leukemia; AML acute myelogenous leukemia; ATRA, all-*trans* retinoic acid; AsO_3, arsenic trioxide; droplet-RT-PCR, droplet-reverse transcription-polymerase chain reaction; FISH, fluorescence in situ hybridization; IQ, instant quality; RT-qPCR, quantitative RT-PCR; PB, peripheral blood; BM, bone marrow.

1. Introduction

Acute promyelocytic leukemia (APL) constitutes 5–8% of all cases of adult acute myelogenous leukemia (AML) [1]. The presence of the *PML-RARA* fusion gene, which results from a balanced reciprocal translocation, i.e., t(15;17)(q22;q21), is the molecular hallmark of APL. APL is a highly aggressive disease presenting hemorrhagic complications from a characteristic coagulopathy [2]. All-*trans* retinoic acid (ATRA) with chemotherapy or ATRA with arsenic trioxide (AsO_3) is effective and yields higher cure rates with complete remission than previous therapies. ATRA and AsO_3 have specific reactivity for different parts of the PML-RARA fusion protein [3], which serves as the molecular basis for highly effective molecular targeted therapies [1,4-6].

Rapid detection and quantitative assessment of the *PML-RARA* fusion gene, carried out using reverse-transcription polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH), influence the prognoses of the patients with APL. Quantitative RT-PCR (RT-qPCR) is more sensitive than FISH for detection of the fusion gene; however, the expression level of fusion gene by RT-qPCR does not indicate the ratio of cells having the fusion gene to the total number of APL cells. On the other hand, FISH analysis can show the ratio of cells having the fusion gene by counting abnormal signals as needed during the treatment course.

Unfortunately, conventional analysis by RT-PCR and FISH requires one to two days. Therefore, given the high efficacy of ATRA and AsO_3 therapies for patients with APL having the *PML-RARA* fusion gene, it is important to diagnose APL as soon as possible in order to improve patient outcomes and quality of life.

. In this study, we developed a systematic rapid identification/quantification assay for the *PML-RARA* fusion gene using droplet-RT-PCR and instant quality (IQ) FISH (IQ-FISH).

2. Materials and Methods

2.1. Materials

Peripheral blood (PB) and bone marrow (BM) samples from five patients with APL and three controls were used in this study (Table 1). Conventional semi-nested RT-PCR and FISH confirmed that all samples from the patients had the *PML-RARA* fusion gene, whereas all samples from the controls did not contain the fusion gene. Two patients (nos. 1 and 2) were included in our previous study [7]. This study was approved by the Institutional Review Board of Shinshu University (No. 351).

2.2. RNA extraction

Total RNA was extracted from PB or BM samples using a QIAamp RNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

2.3. One-step droplet-RT-PCR

The primers and TaqMan probes for the *PML-RARA* fusion gene were designed as previously reported [8]. The one-step RT-PCR reaction mixture contained RNA, SuperScript III/Platinum Taq Mix (Life Technologies, Grand Island, NY), reaction buffer composed of Tris–HCl pH 9.0, KCl and $MgCl_2$, 0.8 μmol/L of each primer, and 0.2 μmol/L of the TaqMan probe in a final volume of 10 μL. One microliter of each reaction mixtures was used for the droplet-PCR assay. The reaction conditions used in the present study were as follows: 50°C for 300 s for RT, 95°C for 10 s for inactivation of the reverse transcriptase, and 50 cycles at 95°C for 5 s and 62°C for 25 s. In this study, we set an arbitrary standard for evaluating the amplification; samples were considered positive when the fluorescent level of amplification was more than 2.0.

2.4. Conventional FISH

Conventional FISH was performed using Vysis LSI Hybridization buffer and Vysis LSI Dual-color, Dual-Fusion Probe (Abbott Molecular/Vysis. Des Plaines, IL) as previously described [9]. We scored 500 nuclei from PB or BM samples to determine the number of yellow fusion signals using a fluorescence microscope (Axio Imager Z2) equipped with ISIS system software (MetaSystemes Hard & Software, Althlussheim, Germany).

2.5. IQ-FISH

IQ-FISH analysis was performed using an IQ-FISH Fast Hybridization Buffer, Sure FISH *PML-RARA* Dual fusion probe (Agilent Technologies , West Cedar Creek, TX) and a Dako Cytology FISH Accessory Kit (Dako, Glostrup, Denmark) containing Pretreatment buffer, Wash buffer and Stringent wash buffer. The sample slides were chemically aged by treatment with pretreatment buffer at 95°C for 10 min and then cooled at room temperature for 10 min. The slides were dehydrated through an ethanol series (70%, 85%, and 100%), and the probe mixture was then applied. The slides were sealed with coverslips, denatured at 66°C for 10min, and incubated at 45°C for 1 h. Thereafter, the slides were washed with Wash buffer and Stringent wash buffer according to the manufacturer's instructions. The nuclei were stained and mounted with DAPI II counterstain (Abbott Molecular/Vysis. Des Plaines, IL) and VECTASHIELD Mounting Medium （Vector Laboratories, Burlingame, CA） . The signals obtained by IQ FISH were analyzed as described for conventional FISH.

3. Results

3.1. Droplet-RT-PCR for detection of the *PML-RARA* fusion gene

The *PML-RARA* fusion gene was detected by droplet-RT-PCR in all samples from the five patients with APL within 26 min (Fig. 1). No amplification was observed from control samples. Long-type and short-type fusion genes were amplified from four and one samples, respectively. Short-type of *PML-RARA* (patient no. 5) was amplified faster than the long-type of *PML-RARA* (patient nos. 1-4).

3.2. IQ-FISH and conventional FISH

The IQ-FISH and conventional FISH protocols were completed within 4 h in which probe hybridization time was 1 h, and 2 days in which probe hybridization time was 18 h, respectively. IQ-FISH yielded yellow fusion signals from all samples from patients with APL, showing the same strong, clear signals as obtained by conventional FISH (Fig. 2). There were no significant differences in positive signal percentage obtained by IQ-FISH and conventional FISH (Table 1).

4. Discussion

In this study, we developed a rapid assay system for detecting and quantifying the *PML-RARA* fusion gene by using droplet-RT-PCR and IQ-FISH simultaneously. This molecular/cytogenetic assay system, combined with morphological examination, was completed within 4 h (Fig. 3); morphological examination required 1 h, droplet-RT-PCR required 1h (with an RT-PCR time of 26 min), and IQ-FISH required 4 h (with a probe hybridization time of 1 h). Droplet-RT-PCR and IQ-FISH compensate the morphological examination and would allow patients to start molecular-targeted therapies with ATRA and AsO_3 more quickly than conventional analyses.

RT-PCR is a robust method that can be used to confirm the presence of the *PML-RARA* fusion gene. APL is initially diagnosed by morphologic examination of blood smears; however, morphological examination alone cannot provide molecular information required for effective therapy. Thus, detection of the *PML-RARA* fusion gene is important for selecting an effective molecular-targeted therapy, and RT-PCR can be used for this purpose, even in the case of cryptic *PML-RARA* rearrangement without t(15; 17) on G-banding [10].

RT-PCR can detect the *PML-RARA* fusion gene faster than the other molecular/cytogenetic methods such as G-banding and FISH; however, conventional RT-PCR or RT-nested PCR requires about 2–6 h. Additionally, APL can be morphologically diagnosed within 1 h by examining PB and/or BM smears subjected to May-Grunwald-Giemsa, peroxidase, or esterase staining. We developed the rapid droplet-RT-PCR method, which can detect both long-type and short-type *PML-RARA* fusion genes within 26 min. Thus, total time of droplet-RT-PCR from sample collection to amplification was 1 h, and the results could provide a rapid molecular basis for therapeutic decision-making compared with the time required for morphological examination.

FISH is a well-establish tool for detection of translocations by cytogenetic analysis and can quantitatively calculate the number of cells with translocations by counting the fluorescent signals in the nuclei of interphase cells. RT-qPCR, FISH, and morphological examinations provide quantitative information for the APL cells. Among these methods, FISH can more correctly evaluate the percentage of cells harboring the fusion gene than other methods. However, conventional FISH requires at least 4–6 h to obtain appreciable FISH signals [11], and the slides are usually incubated overnight in formamide-based hybridization buffer for hybridization. Several studies have examined the possibility of reducing the hybridization time [11–13]. Microwave treatment, which applies short beams to the slide after the denaturation step, reduces the hybridization time to 30 min; however, fine-tuning of the microwave pulse is needed to avoid overheating [12].

Recently, the IQ-FISH method has emerged as a potential alternative to conventional FISH. This method uses an ethylene carbonate buffer instead of formamide-based buffer for hybridization. The ethylene carbonate buffer has a lower affinity to the bases through hydrogen bonds than formamide, markedly shortening hybridization time. In addition, the ethylene carbonate buffer can be used at a lower denaturation temperature which reduces background staining [14,15]. Labor and intensive manipulations are not necessary for IQ-FISH, and this method has been introduced as a routine test in clinical laboratories without the requirement for special equipment. In this study, the IQ-FISH showed the same clear signals as conventional FISH with overnight hybridization. Moreover, there were no significant differences in signal ratios for rapid IQ-FISH and conventional FISH. IQ-FISH allowed cytogenetic quantitative evaluation of the *PML-RARA* fusion gene within 4 h of receiving the sample.

In conclusion, simultaneous droplet-RT-PCR and IQ-FISH, combined with morphological examination of blood smears, is one of the most reliable systems for the rapid diagnosis of APL with the *PML-RARA* fusion gene. This system diagnoses a patient as having APL with the *PML-RARA* fusion gene within 4 h, allowing effective therapy with ATRA and AsO3 to be started immediately

References

[1] Mi J. Current treatment strategy of acute promyelocytic leukemia. Front Med 2011; 5:341-7.

[2] Stein E, Mcmahon B, Kwaan H, Altman JK, Frankfurt O, Tallman MS. The coagulopathy of acute promyelocytic leukaemia revisited . Best Pract Res Clin Haematol 2009;22:153-63

[3] Lallemand-Breitenbach V, de Thé H. Retinoic acid plus arsenic trioxide, the ultimate panacea for acute promyelocytic leukemia? Blood 2013;122:2008-10.

[4] Tallman MS, Altman JK. How I treat acute promyelocytic leukemia. Blood 2009; 114:5126-35.

[5] Kuhnl A, Grimwade D. Molecular markers in acute myeloid leukemia. Int J Hematol 2012;96:153-63

[6] Neame PB, Soamboonsrup P, Leber B, Carter RF, Sunisloe L, Patterson W, et al. Morphology of acute promyelocytic leukemia with cytogenetic or molecular evidence for the diagnosis: characterization of additional microgranular variants. Am J Hematol 1997;56:131-42

[7] Sueki A, Matsuda K, Taira C, Yamaguchi A, Koeda H, Takagi F, et al. Rapid detection of *PML-RARA* fusion gene by novel high-speed droplet-reverse transcriptase-polymerase chain reaction: Possibility for molecular diagnosis without lagging behind the morphological analyses. Clin Chim Acta 2013;415:276-8

[8] Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - A Europe Against Cancer Program. Leukemia 2003;17:2318-57

[9] Matsuda K, Hidaka E, Ishida F, Yamauchi K, Makishima H, Ito T, et al. A case of acute myelogenous leukemia with MLL-AF10 fusion caused by insertion of 5' MLL into 10p12, with concurrent 3' MLL deletion. Cancer Genet Cytogenet. 2006;171:24-30.

[10] Han JY, Kim KE, Kim KH, Park JI, Kim JS. Identification of *PML-RARA* rearrangement by RT-PCR and sequencing in an acute promyelocytic leukemia without t(15;17) on G-banding and FISH. Leuk Res. 2007;31:239-43.

[11] Kolhe R, Mangaonkar A, Mansour J, Clemmons A, Shaw J, Dupont B, et al. Utility and impact of early t(15;17) identification by Fluorescence In Situ Hybridization (FISH) in clinical decision making for patients in Acute Promyelocytic Leukemia (APL). Int J Lab Hematol 2015; 37:515-20

[12] Soriani S, Mura C, Panico AR, Scarpa AM, Recchimuzzo P, Dadati R, et al. Rapid detection of t (15; 17)(q24; q21) in acute promyelocytic leukaemia by microwave‐assisted fluorescence in situ hybridization. Hematol Oncol 2015;3:259-63

[13] Ridderstråle KK, Grushko TA, Kim HJ, Olopade OI. Single-day FISH procedure for paraffin-embedded tissue sections using a microwave oven. Biotechniques 2005;39:316-20.

[14] Matthiesen SH, Hansen CM. Fast and Non-Toxic In Situ Hybridization without Blocking of Repetitive Sequences. PLOS One 2012;7:7

[15] Tafe LJ, Steinmetz HB, Allen SF, Dokus BJ, Tsongalis, GJ. Rapid fluorescence in situ

hybridisation (FISH) for HER2 (ERBB2) assessment in breast and gastro-oesophageal cancer. J Clin Pathol 2015;68:4:306-8

Figure Captions.

Figure 1. Droplet-RT-PCR for detecting the *PML-RARA* fusion gene

The PML-RARA fusion gene was amplified in all samples obtained from the five patients with APL (long-type PML-RARA, patients nos 1–4; short-type PML-RARA, patient no 5). The fusion gene was not detected in control samples (C1–C3). Fluorescence levels over 2.0 (dotted lines) were considered positive amplification. X axis: cycles and time of PCR, Y axis: fluorescence level (arbitrary units).

Figure 2. Fluorescence signals of the *PML-RARA* fusion gene by conventional FISH and IQ-FISH.

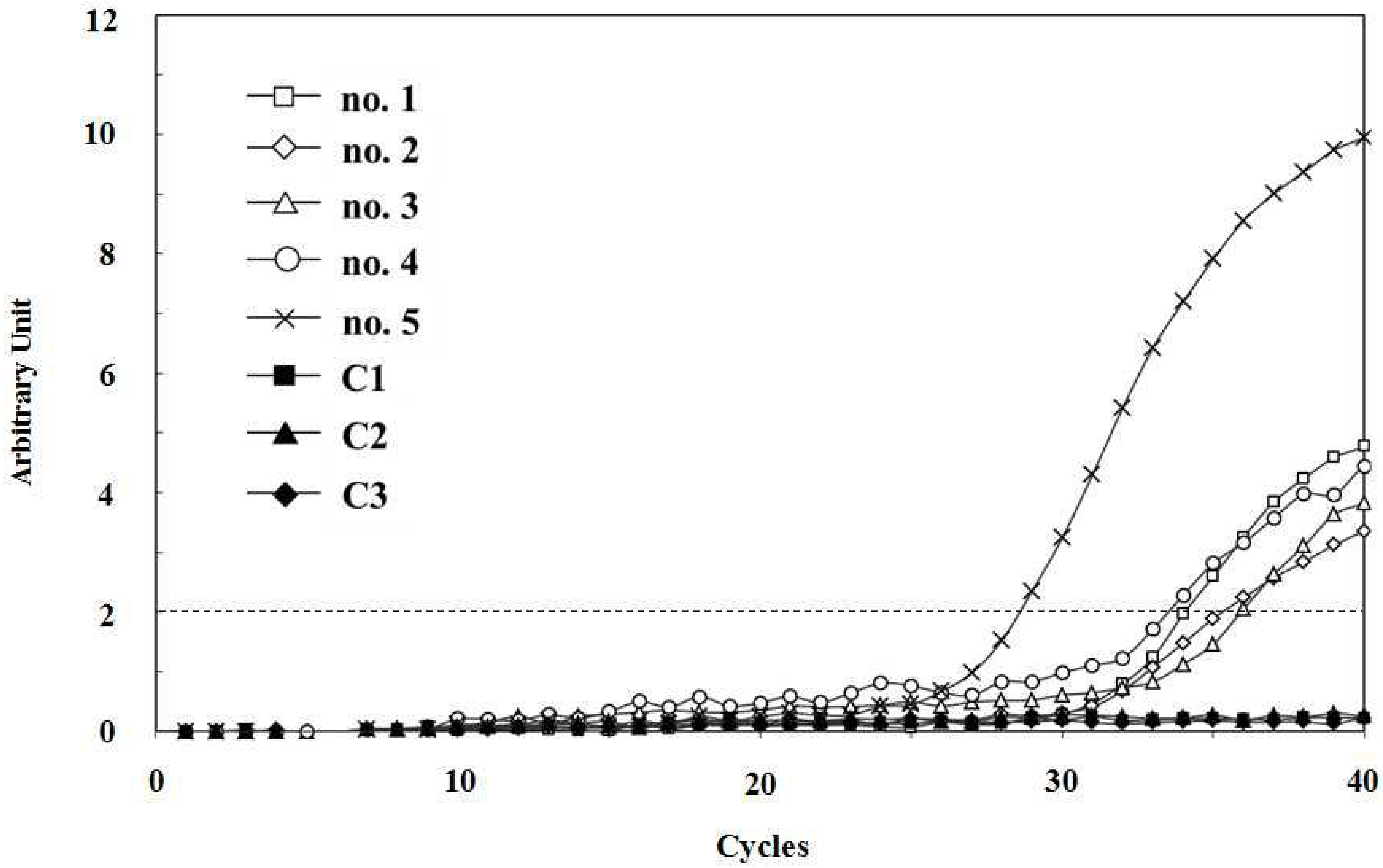
Fluorescence signals representing the *PML-RARA* fusion gene were obtained by conventional FISH (A) and IQ-FISH (B). Red signal: the normal chromosome 15 (PML), green signal: the normal chromosome 17 (RARA), yellow fusion signals (arrows): derivative chromosomes involved in t(15;17)(q24;q21)..

Figure 3. A recommended workflow for diagnosing APL with the *PML-RARA* fusion gene

Table 1. Clinical and laboratory data from five patients with APL

Patient no.	Sex	Age (years)	Sample type	FISH signal for *PML/RARA* (%)		Type of fusion gene	Karyotype
				Conventional FISH	IQ FISH		
1	Male	38	PB	86.8	85.0	(+) Long type	No growth
2	Female	74	BM	85.8	86.8	(+) Long type	46,XX,t(15;17)(q22;q21)[3]/46,XX[15]
3	Male	71	BM	81.0	79.0	(+) Long type	46,XY,add(11)(q11.2),t(15;17)(q22;q21)[3]/46,XY[11]
4	Male	63	BM	91.4	90.0	(+) Long type	46,XY,t(15;17)(q22;q21)[18]/46,XY[2]
5	Male	48	BM	95.0	96.4	(+) Short type	46,XY,t(15;17)(q22;q21)[7]/46,XY[5]

PB, peripheral blood; BM, bone marrow; IQ FISH, instant quality fluorescence in situ hybridization; Long type, the long form of the *PML-RARA* mRNA transcript (breakpoint in intron 6 of *PML*); Short type, the short form of the *PML-RARA* mRNA transcript (breakpoint in intron 3 of *PML*). Patients 1 and 2 were also used in our previous study [7].



12
10
8
6
4
2
0
Arbitrary Unit
0
10
20
30
40
Cycles
no. 1
no. 2
no. 3
no. 4
no. 5
C1
C2
C3

Fig 1

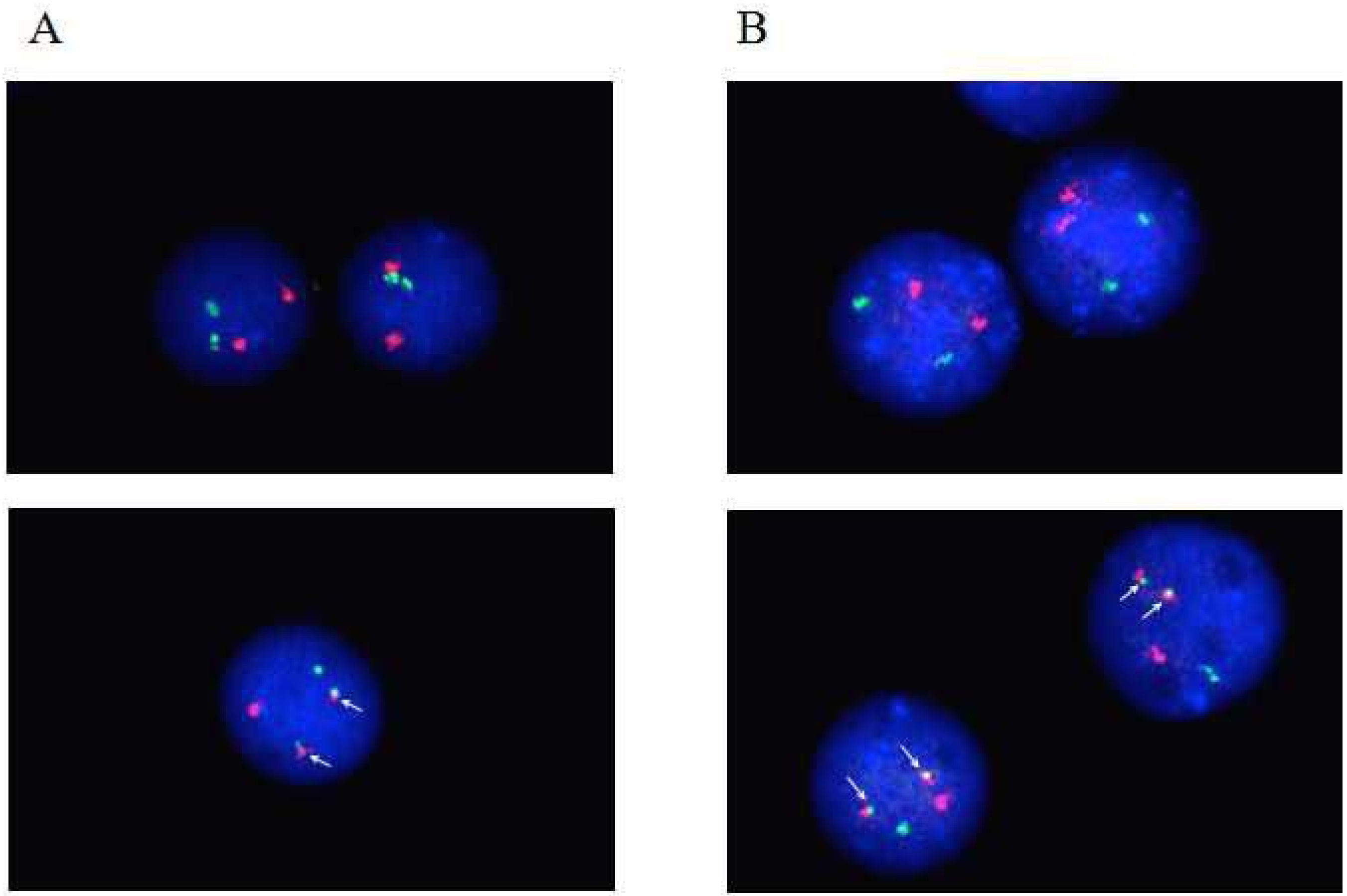


Fig 2

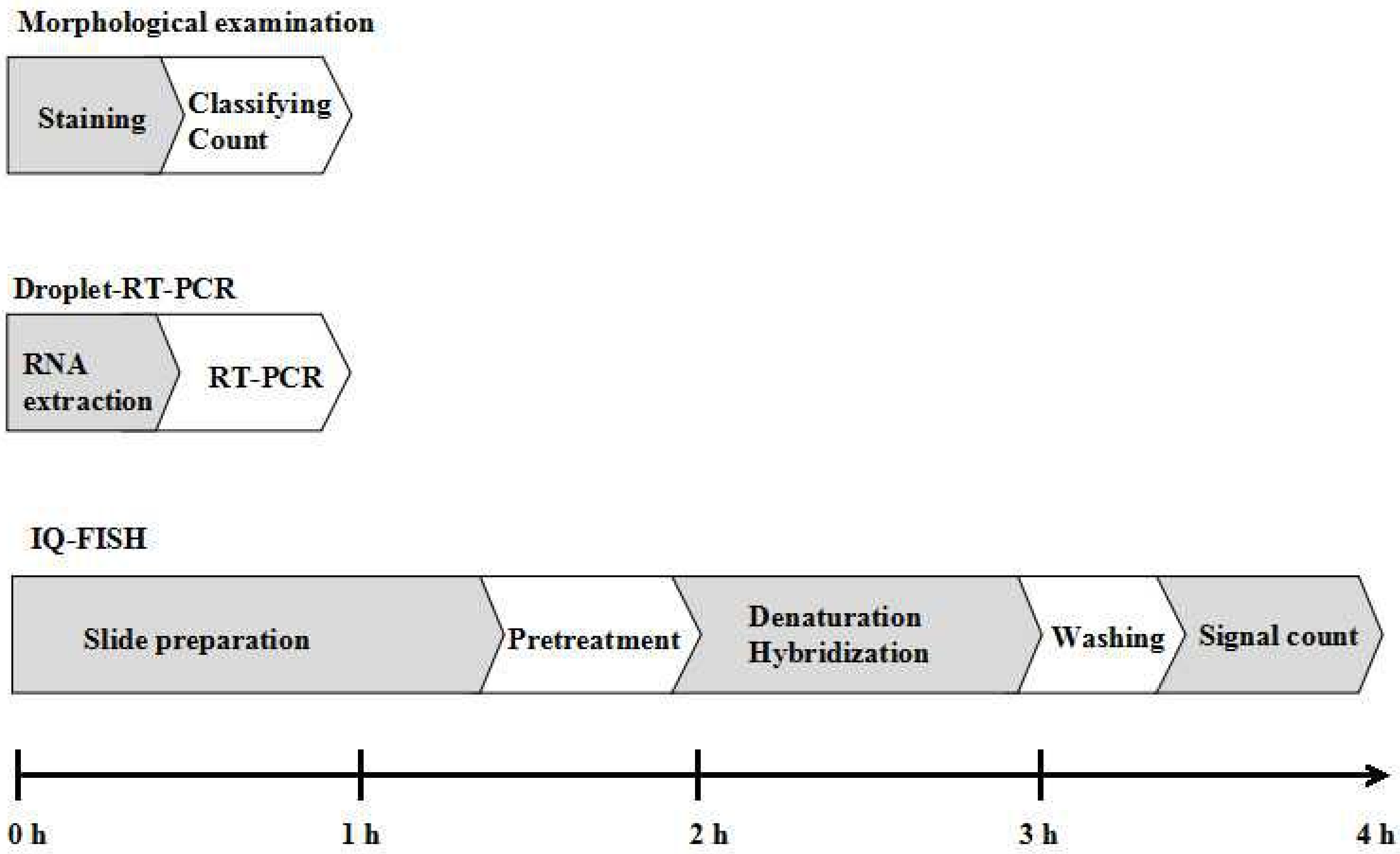

Morphological examination
Staining
Classifying
Count
Droplet-RT-PCR
RNA
extraction
RT-PCR
IQ-FISH
Slide preparation
Pretreatment
Denaturation
Hybridization
Washing
Signal count
0 h
1 h
2 h
3 h
4 h

Fig 3