J Ind Microbiol Biotechnol (2012) 39:1161–1168
DOI 10.1007/s10295-012-1118-9

BIOTECHNOLOGY METHODS

A novel and efficient assay for identification and quantification of *Acidithiobacillus ferrooxidans* in bioleaching samples

Shoushuai Feng · Yu Xin · Hailin Yang · Ling Zhang · Wenliang Kang · Xiaole Xia · Wu Wang

Received: 19 December 2011 / Accepted: 29 February 2012 / Published online: 20 March 2012
© Society for Industrial Microbiology and Biotechnology 2012

Abstract *Acidithiobacillus ferrooxidans* is a Gram-negative, acidophilic, and chemolithotrophic bacterium that is active in bioleaching. The leaching efficacy is directly influenced by the biomass changes of this specie in bioleaching microbial community. In order to perform a simple and sensitive assay on *A. ferrooxidans* from mixed strains in this process, a novel assay was developed based on sandwich hybridization assay with the aid of S1 nuclease treatment and fluorescent labeling. In the work, a designed DNA probe complementary to the conservative region of its 16S rRNA was synthesized, which showed high accuracy for distinguishing homologous species with the exclusion of even-only two base pairs difference. The specificity of this assay was verified in different systems with mixed strains, and the quantitative result was proved by comparison of microscopic cell counting. The detection sensitivity was about 8×10^2 cells/ml and the inter-assay coefficient of variation of three independent assays was from 3.8 to 7.7 %, respectively. In addition, the cycle of assay was about 3–4 h when the cost estimated was less than $0.5 per sample. This assay method might be applied for identifying and monitoring any kind of bacterial strain from a mixed microbial flora in bioleaching or other areas.

Keywords Quantitative assay · Sandwich hybridization · 16S rRNA · *Acidithiobacillus ferrooxidans* · Bioleaching

S. Feng · Y. Xin · H. Yang · L. Zhang · W. Kang · X. Xia · W. Wang (✉)
The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, People's Republic of China
e-mail: bioprocessor@yahoo.cn

Introduction

It has become urgent to make full use of mineral resources with the over-exploitation of high-grade ores in the past few decades [5, 19]. Compared to the traditional smelting method, bioleaching is considered a green metallurgical technology with its characteristics of less operating costs, lower investment in infrastructure, and higher recovery [7]. The commonly used microbial species in bioleaching processes might involve *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, *Acidithiobacillus thiooxidans*, and *Acidithiobacillus caldus*, etc. *A. ferrooxidans* is a Gram-negative, acidophilic, and chemolithotrophic bacterium that obtains energy from the oxidation of ferrous iron (Fe[II]) and reduced sulfur compounds [1, 9]. It is the first reported and model bacterium and the most broadly studied bacterium in the bioleaching process, especially with the release of the genome sequence [23]. More studies have revealed that the success of industrial bioleaching is greatly influenced by the biomass changes of *A. ferrooxidans* in the microbial community. Therefore, it is of great importance to monitor and control its populations in different stages for improving leaching efficacy [24]. However, a limited number of studies provide a qualitative and quantitative description of *A. ferrooxidans* populations in the complex bioleaching systems.

In the present work, microscopic counting, colony cultivation, immunological and nucleic-acid based molecular techniques are currently used for identification or quantification of *A. ferrooxidans* [10, 25]. Microscopic counting could quantify total cells but is helpless in identification. It is difficult to distinguish *A. ferrooxidans* from *A. thiooxidans* or *A. caldus* in a bioleaching system with similar morphology [4]. With cultivation on solid media, 2–3 weeks of incubation are needed for identification of *A. ferrooxidans*, which is

impossible to monitor bioleaching operations in time [17]. With immunological technology like ELISA, and despite its simple operation procedure, few studies have yet reported using this technology to detect *A. ferrooxidans* in bioleaching because of its unsatisfying stability and accuracy [13]. Besides, species-specific antibody, a crucial material in this technology, is generally expensive and hard to obtain [22]. Identification and quantification of *A. ferrooxidans* could also be rapidly and accurately performed by real-time PCR based on the online fluorescence detection of labeled amplification. *Acidithiobacillus* (66.78 %), *Leptospirillum* (28.29 %), *Sulfobacillus* (3.29 %), and *Ferrimicrobium* (1.64 %) have been observed by 16S rDNA clone libraries and real-time PCR in a commercial non-aeration copper bioleaching heap [8]. However, this technique faces problems in high-efficiency purification and expense [3, 12].

The reasons mentioned above encourage the development of a novel assay, which is characterized as 16S rRNA-based sandwich hybridization assay for detecting a model strain *A. ferrooxidans* in mixed strains system with high sensitivity and reliability.

Materials and methods

Strains and cell lysis

Strains and homologies with *A. ferrooxidans* strain CUMT-1 are listed in Table 1. *A. ferrooxidans* strain CUMT-1 (*A. ferrooxidans*) and *Leptospirillum ferrooxidans* strain TLS-3 (*L. ferrooxidans*) were grown in 9 K media; *Acidithiobacillus thiooxidans* strain ZJJN-3 (*A. thiooxidans*) and *Acidithiobacillus caldus* strains ZJJN-6 (*A. caldus*) were grown in Starky-S media [24]. Also, 10 % chalcopyrite was added for bioleaching. Chalcopyrite was obtained from Tonglingshan (Anhui, China). The mineral was ground and sieved through a 200-mesh grid, and the diameter of the particles was <75 μm.

Supernatant of samples was collected by centrifugation at 380 × *g* for 2 min. Cells were harvested by centrifugation at 12,000 × *g* for 10 min. The chosen method for cell lysation was 10 ml lyses buffer (80 % formamide, 450 mM NaCl, 5 mM Na_2EDTA, 1 mg/ml yeast tRNA, 1 % SDS, pH 6.4). Samples were sonicated at 50 % duty cycle and 300-W output for 4 min. The cell lysates were stored at –20 °C.

Design of DNA probes

The 16S rDNA sequence of *A. ferrooxidans* CUMT-1 was blasted in GenBank (http://www.ncbi.nlm.nih.gov/.), and special region different with other bioleaching microorganisms was identified. A segment of DNA (named probe A.f-896) was designed complementary to the conservative region (896–964) of 16S rRNA from *A. ferrooxidans*; capture probe (CP) was complementary to the 3′ terminal region of probe A.f-896; signal probe (SP) was complementary to the 5′ terminal region of probe A.f-896 (Table 2). These probes were synthesized at Sangon Biotech (Shanghai, China).

Immobilization of capture probe

A total of 100 μl of 5 μg/l streptavidin (Takara, Japan) dissolved in carbonate buffer (0.05 mol/l, pH 9.6) was added into a microwell and sealed with parafilm. Each well was washed three times with PBS-Tween 20 (0.01 M PBS, 0.5 % Tween 20, pH 7.2) and then 100 μl 0.1 % bovine serum albumin dissolved in PBS (0.01 M, pH 7.2) was added to each well. These wells were washed as above after being incubated at 37 °C for 30 min. Next, 100 μl of 10 nM biotin-labeled CP dissolved in PBS was added into each well coated. Finally, these wells were washed as above

Table 1 Strains used in the present study

Species	Strain	Homologies with *A. ferrooxidans*/%	Collection site
A. ferrooxidans	CUMT-1	100	Mining University of China, Jiangshu, China
A. thiooxidans	ZJJN-3	98	Zhijinshan acid mine water, Fujian, China
A. caldus	ZJJN-6	95	Zhijinshan acid mine water, Fujian, China
L. ferrooxidans	TLS-3	81	Tonglingshan acid mine water, Anhui, China

Table 2 Probes for detecting *A. ferrooxidans* designed in the present study

Probe	Sequence	Modification	GC/%	Length/bp
Capture probe	5′-ATCTCTGCAGAATTCCGGACATGTCAA-3′	5′-biotin	44.4	27
Signal probe	5′-TCGCGTTGCATCGAATTAAACCACAT-3′	3′-fluorescein	40.7	27
Probe A.f-896	5′-TGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACA TGTCCGGAATTCTGCAGAGAT-3′	None	46.3	69

after being incubated at 37 °C for 30 min. The microwells were stored at 4 °C until use.

Steps of 16S rRNA-based sandwich hybridization assay

A total of 45 of μl cell lysates, 5 μl of 100 nM probe A.f-896 solution, and 50 μl of mineral oil were transferred to a centrifuge tube and then heated at 94 °C for 15 min and incubated at 30 °C for 1 h. Then, 50 μl of S1 nuclease buffer (Sang Biotech, China; 60 units S1 nuclease in 1.4 M NaCl, 22.5 mM $ZnSO_4$, 250 mM CH_3COONa, pH 4.5) was added into each tube and incubated at 37 °C for 20 min. After adding 200 μl of stop solution (62.5 mM NaOH, 30 mM EDTA and 0.5 M PBS, pH 7.2), the tubes were heated to 94 °C for 15 min and then cooled to room temperature (25 °C) for 5 min. An amount of 100 μl of the mixed solution above and 100 μl of 10 nM SP dissolved in hybridization buffer (4× SSC, 10 % formamide, 0.02 % SDS, pH 7.2) were added into each well coated with CP, which was then covered with 50 μl of mineral oil. Finally, these wells were washed as above after being incubated at 45 °C for 30 min.

We then added 100 μl of anti-fluorescein–horse radish peroxidase (Takara, Japan; 1:5,000 diluted in PBS, 0.1 % bovine serum albumin) into each well for adsorption with SP and the wells were incubated at 37 °C for 15 min and washed as noted above. Finally, 100 μl of TMB solution (0.1 M citric acid, 0.2 M Na_2HPO_4, 0.1 % H_2O_2, 0.01 % TMB) was added into each well [13]. 50 μl of 2 M H_2SO_4 was added for stopping the color reaction after incubation at 37 °C for 10 min. Then $A_{450\ nm/630\ nm}$ was measured.

Detection of *A. ferrooxidans* by 16S rRNA-based sandwich hybridization assay and microscopy

A. ferrooxidans, *A. thiooxidans*, *L. ferrooxidans*, and *A. caldus* were employed for testing the specificity and sensitivity of 16S rRNA-based sandwich hybridization assay (Table 3). One-milliliter cultures of *A. ferrooxidans, L. ferrooxidans*, *A. caldus*, and *A. thiooxidans* at logarithmic phase were removed from culture media, respectively. Before microscopic counting, samples from bioleaching system were centrifuged at 380 × *g* for 2 min to get rid of solid particles and impurities.

Table 3 Samples for comparison between 16S rRNA-based sandwich hybridization assay and microscopic cell counting

Sample	*A. ferrooxidans*	*A. thiooxidans*	*L. ferrooxidans*	*A. caldus*
1	5.2×10^3	–	–	–
2	–	5.2×10^3	–	–
3	–	–	5.2×10^3	–
4	–	–	–	5.2×10^3
5	5.2×10^3	5.2×10^3	5.2×10^3	5.2×10^3
6	–	5.2×10^3	5.2×10^3	5.2×10^3
7	2.6×10^3	2.6×10^3	2.6×10^3	2.6×10^3
8	1.3×10^4	1.3×10^4	1.3×10^4	1.3×10^4
9	–	–	–	–

Different designed systems were divided into a single-strain system (sample 1–4) and a mixed-strains system (sample 5–8). Sample 9 was blank control without any cells. The numbers representing cells densities were counted three times in the single-strain system, respectively, by microscopy

Results and discussion

Principle of 16S rRNA-based sandwich hybridization assay

It is the first time that *A. ferrooxidans* in a bioleaching system has been detected using this 16S rRNA-based sandwich hybridization assay. The schematic consisted of three stages: nuclease error correction, immobilization of CP, and signal transfer and detection (Fig. 1). In the stage of nuclease error correction, the first hybridization was processed between probe A.f-896 and 16S rRNA in crude cell lysates. The unstable 16S rRNA signal was converted to the same amount of DNA signal. Unbound and imperfectly matched probes A.f-896 were digested by S1 nuclease. In the stage of immobilization of CP, CP was immobilized at the bottom of the well by a stable non-covalent bond between streptavidin and biotin. These two stages could be processed simultaneously in the same condition and the time consumption of the assay was significantly reduced. In the stage of signal transfer and detection, the second hybridization was processed between probe A.f-896 and CP when the third hybridization was processed between probe A.f-896 and SP. Then polyclonal antibody-HRP against fluorescein was added for combining with 3′ fluorescein-linked SP. Unbound probes or compounds in each stage were washed away as described in the Materials and methods section. Finally, the fluorescent signal intensity was measured by a common microplate reader though color reaction between the HRP and TMB system.

The hybridization specificity of probe A.f-896

Probe A.f-896 was blasted with relevant sequences of other three common bioleaching bacteria as *L. ferrooxidans*, *A. thiooxidans*, and *A. caldus* for testing its specificity. As shown in Fig. 2, there were at least two base pairs of probe A.f-896 different with other three strains. The real diversity between the chosen 69-base region and the relevant segments in three other bioleaching bacteria were as follows: *L. ferrooxidans* 21.7 % (15 base pairs), *A. thiooxidans* 2.9 % (two base pairs) and *A. caldus* 2.9 % (two base pairs). It was reported that oligonucleotide probe could

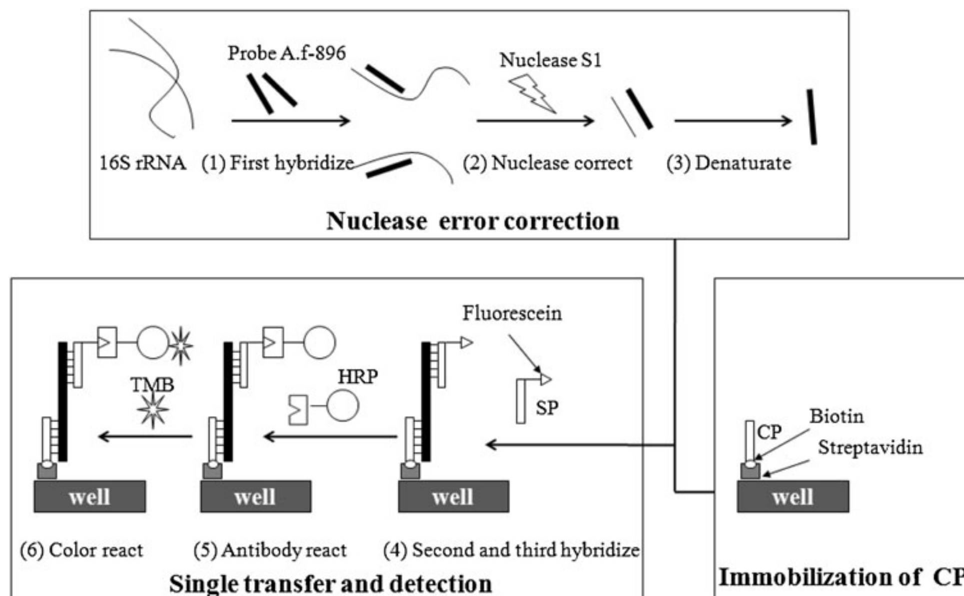

Fig. 1 Schematic of 16S rRNA-based sandwich hybridization assay

1 ATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTCTGGAATCCTGCAGAGAT

2 ATGTGGTTTAATTCGACGCAACGCGAGGAACCTTACCTAGGCTTGACATGTGGTCAGTAGCGAACCGAA

3 ATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTCCGGAACCCTGCAGAGAT

4 ATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTCCGGAATTCTGCAGAGAT

Fig. 2 Sequence of probe A.f-896 blasted with the target 16S rRNA region. The *highlighted letters* on a black background represent the mismatch sites with the probe A.f-896. *1 A. thiooxidans*, *2 L. ferrooxidans*, *3 A. caldus*, *4* probe A.f-896

distinguish the similar sequences with the exclusion of even only two base pairs difference by the high specificity of nuclease error correction assay [16]. In addition, the G + C % of probe A.f-896 was 46.3 % when those of CP and SP were 44.4 and 40.7 %, respectively. It was suggested that hybridization was processed easily when the G + C % of the probe is 40–60 %, especially when the proportion was nearly between the forward and reverse primers [14].

Optimal conditions for 16S rRNA-based sandwich hybridization assay

Some operating conditions were explored before detecting samples. Optimal probe concentrations were tested for reducing the cost of material. The results showed that signal strength increased in an S-shaped curve with the growth of concentration (Fig. 3). As the concentration of probe CP or SP increased from 1 to 10 nM, the signal rapidly intensified. When the concentration was lower than 1 nM, the detection signal was weak; when it was higher than 10 nM, the absorbance changed little. This might be because the folded structure was easy to be formed and some binding sites might be prevented under too high concentration [2]. In summary, 10 nM was chosen as the work concentration of CP and SP; 100 nM was determined as the work concentration of probe A.f-896 to ensure probe A.f-896 molecules exceed the amount of targeting sequences.

Optimal hybridization time was tested for reducing the cycle of assay. During hybridization of probe A.f-896 with 16S rRNA, when the hybridization times were 10 min, 0.5, 1, 1.5, and 3 h, the hybridization efficiencies were 30.7, 41.3, 91.3, 97.3, and 83.3 %, respectively. Over 1 h of hybridization time might be associated with degradation of DNA probes and time consuming in future application in future application [25]. Therefore, 1 h was chosen as the hybridization time of probe A.f-896 with 16S rRNA in our protocol. Additionally, 0.5 h was chosen as the hybridization time in the second and third hybridization phases, respectively (Fig. 4a).

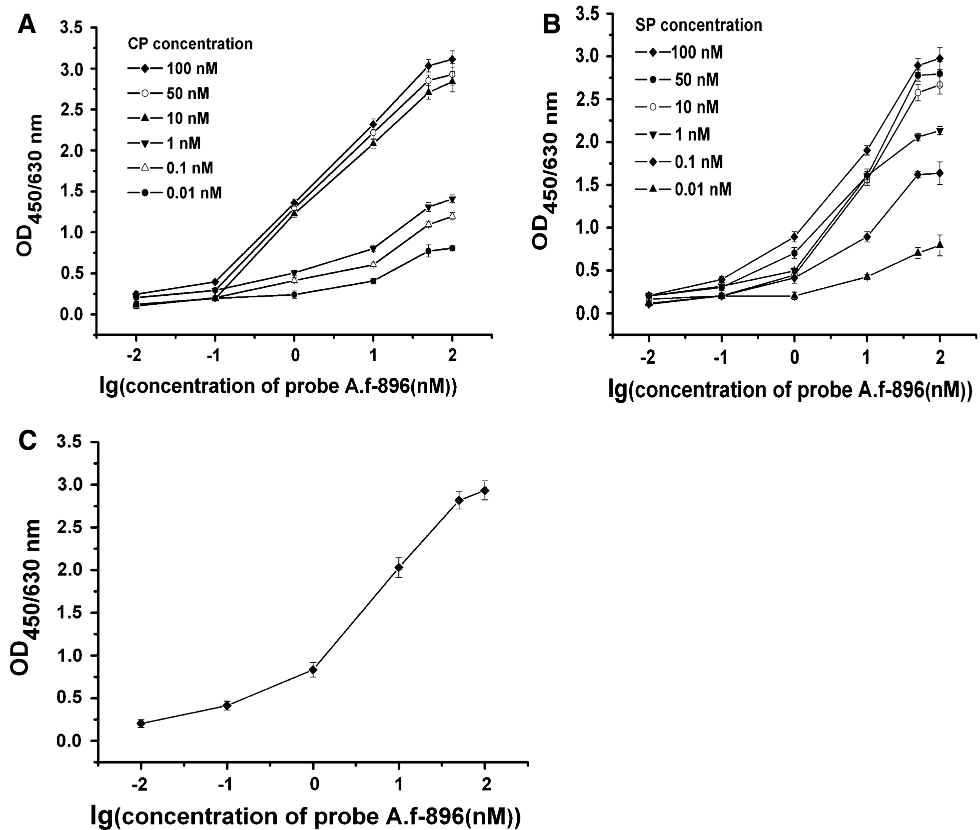

Fig. 3 Effects of probe concentrations on 16S rRNA-based sandwich hybridization assay. **a** Capture probe (CP) concentrations. **b** Signal probe (SP) concentrations. **c** Probe A.f-896 concentrations with 10 nM CP and 10 nM SP

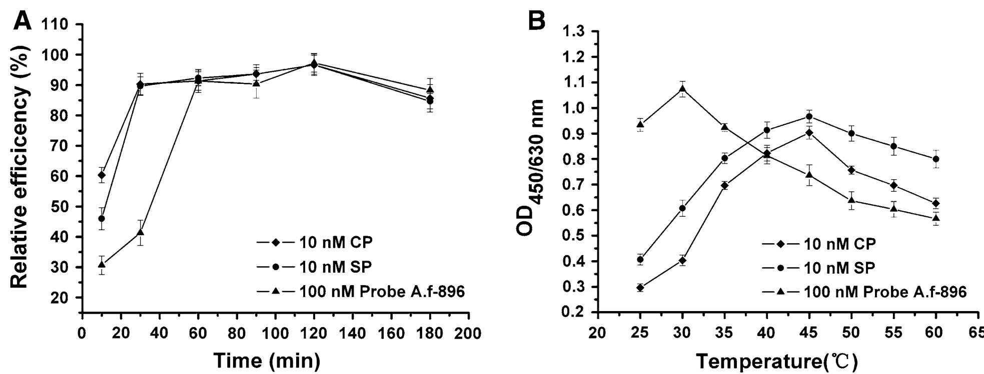

Fig. 4 Effects of time and temperature on 16S rRNA-based sandwich hybridization assay. **a** Time, the hybridization efficiency of 2 h was set as 100 %. **b** Temperature, the concentrations of probe A.f-896, CP and SP in all hybridization were 100, 10, and 10 nM, respectively

Temperature was reported to be able to influence the efficiency of hybridization by changing the molecular motion and secondary structure of the probes [22]. T_m was related with the (G + C) content, probe length, ionic concentration, hybrid system and formamide content, and other factors. Generally, T_m is 25 °C higher than the hybridization temperature [14]. In our protocol, $T = 81.5\ °C + 16.6 \times lgM + 0.41 \times (G + C)\ \% - 500/n - 0.61\ (formamide\ \%) - 25\ °C$; M—molar concentration of Na^+, mol/l; n—length of the probe, mer. Based on the above formula, 29, 46, and 45 °C were figured out as hybridization temperatures of three hybridization phases, respectively, which was basically consistent with the testing results 30, 45, and 45 °C (Fig. 4b).

Calibration curve of 16S rRNA-based sandwich hybridization assay

The calibration curve was established on the basis of the above optimal conditions. The detection equation of the best linear curve was '$y = 0.376x - 0.111$', $R^2 = 0.997$, where x refers to cell density (E3 cells/ml), y refers to the absorbance at 450 nm, referred at 630 nm. The coefficient of correlation reached 0.997 and the inter-assay

coefficient of variation was from 6.8 to 8.5 %, respectively. The limit detection of 0.8×10^3 cells/ml and the linear range was 0.8×10^3 to 2.4×10^3 cells/ml. The density of bacteria and archaea oxidizing metal sulfides, especially *A. ferrooxidans* in mine heaps, was generally high than 1.0×10^5 cells/ml, which was higher than the linear range [18, 26]. The problem could be solved by diluting or concentrating on the basis of estimating cell density of target sample when some bioleaching samples might be out of the linear range.

Qualitative and quantitative detection by 16S rRNA-based sandwich hybridization assay

The specificity and sensitivity of 16S rRNA-based sandwich hybridization assay were tested in different bioleaching systems and are listed in Table 3. Qualification of *A. ferrooxidans* by 16S rRNA-based sandwich hybridization assay is shown in (Fig. 5a). In the single-strain system, the absorbance of sample 1 consisting of *A. ferrooxidans* was 1.2, and it was far greater than the samples without target cells. The absorbance of sample 2 consisted of *A. thiooxidans* and was the greatest in samples without *A. ferrooxidans*. It should be due to the most homologous of *A. thiooxidans* with *A. ferrooxidans* compared to *A. caldus* and *L. ferrooxidans* [19]. However, the influence could be ignored compared to sample 1. In the mixed-strains system, the absorbance of sample 5 dropped slightly from 1.2 to 1.1 compared to sample 1, which indicated a slight decrease in accuracy of this assay in this mixed-strains system. It is owing to that there was more interference such as similar sequences or extracts from different strains slightly influence the accuracy of sandwich hybridization. However, the maximum value of other samples without target strain was only 0.16, and it was still far below the absorbance of sample 5. The result proved that the specificity of probe A.f-896 was accurate for distinguishing sequences with the exclusion of even only two base pairs difference. With different cell densities of *A. ferrooxidans*, the absorbances of sample 1 and 5 were 1.2 and 1.1, respectively, when the cell density of *A. ferrooxidans* was 5.2×10^3 cells/ml; the absorbance of sample 7 was 0.8 when cell density was 2.6×10^3 cells/ml; the absorbance of sample 8 was 1.53 accordingly when the cell density was 1.3×10^3 cells/ml. Samples with different cell densities were detected for testing the stability of this assay after being diluted. The result proved that little interference increased and this assay was still accurate after being diluted.

Quantification of *A. ferrooxidans* by 16S rRNA-based sandwich hybridization assay is shown in (Fig. 5b). With 16S rRNA-based sandwich hybridization assay, 5.1×10^3 and 4.9×10^3 cells/ml were respectively detected in samples 1 and 5; while 2.4×10^3 and 1.25×10^4 cells/ml were detected in samples 7 and 8. They were all consistent with the results of microscopic cell-counting, which were 5.2×10^3, 2.6×10^3, and 1.3×10^3 cells/ml, respectively. The inter-assay coefficient of variation in three independent assays was from 3.8 to 7.7 %, respectively. It also demonstrated that other bioleaching microorganisms had no obvious interfering in quantification of *A. ferrooxidans* in bioleaching with mixed strains.

Compared to other techniques in identification and quantification of *A. ferrooxidans* (Table 4), this assay could detect *A. ferrooxidans* in the crude cell lysates without prior purification of 16S rRNA; it was accurate enough for distinguishing homologous species with the exclusion of only two base pairs difference; the cycle of assay was about 3–4 h and detection sensitivity was about 8.0×10^2 cells/ml; the total cost was less than $0.5 per sample without employing expensive lysozyme, proteinase K, and RNAase. Furthermore, there was little requirement of equipment except for a common microplate reader.

Additionally, this method was developed for the rapid detection of bioleaching bacteria in 9 K medium, and the

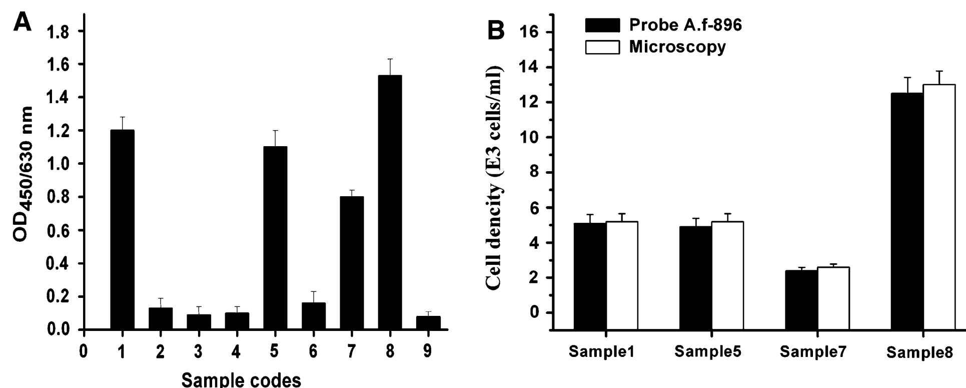

Fig. 5 **a** Identification of *A. ferrooxidans* by 16S rRNA-based sandwich hybridization assay. **b** The comparison between 16S rRNA-based sandwich hybridization assay and microscopic cell counting. Samples are described in Table 3

Table 4 Comparison of proposed techniques for identification and quantification of *A. ferrooxidans*

Techniques	Advantages/application	Disadvantages
Microscopic cell counting	Simple and fast enumeration	Non-quantitative measurement [8]
Cultivation on solid media	Identification of *A. ferrooxidans*; simple and inexpensive cost	Long-term cultivation [9, 15]
Determination of 16S rRNA sequences	Analysis of microbial suspensions with low density of *A. ferrooxidans*	Efficient purification, expensive, and long-term determination [14]
Determination of specific coenzymes	Detection of specific groups of *A. ferrooxidans* in certain conditions	Content of coenzymes is varied depending on species growth conditions [18]
Enzyme-linked immunosorbent assay	Identification of *A. ferrooxidans* with simple operation procedure	Non-quantitative measurement; specific antibody is expensive or unable to be obtained [11]
Fluorescence in situ hybridization	Identification of *A. ferrooxidans* in microbial suspensions by labeling	Non-quantitative measurement; expensive [17, 20]
Real-time PCR	Identification and quantification of *A. ferrooxidans* after purification, amplification, and labeling	Efficient purification, expensive equipment and reagents [6, 8, 21]
RFLP fingerprints of PCR amplified 16S–23S rDNA spacer fragments	Identification and quantification of *A. ferrooxidans* after purification, amplification, and membrane transfer	Efficient purification, complicated operating and expensive [4]
16S rRNA-based sandwich hybridization assay in this study	Identification and quantification of *A. ferrooxidans* in crude cells lysates; inexpensive, little requirement of equipment and no purification needed	Not detected

medium would be improved for imitating more detailed chemical environment in bioleaching when it might be applied in the industrial sites in the future.

In summary, a novel assay based on sandwich hybridization assay with the aid of S1 nuclease treatment and fluorescent labeling was developed for identifying and quantifying *A. ferrooxidans* efficiently in bioleaching system. The procedure and operating conditions are introduced in detail, and the assay results are verified. As a simple and practical technique, the novel assay might provide a practical protocol for identifying and monitoring any kind of bacteria from a mixed microbial flora.

Acknowledgments This work was supported by grants from the National High Technology Research and Development Program of China (863 Program) (No. 2012AA021201), the Program of Innovation Projects Plan of Jiangsu Province (No. CXZZ11_0481), the Scientific Research Foundation for Graduate Students of Jiangnan University (No. JUDCF11013), the Program of the Science and Technology Support Plan of Jiangsu Province (No. SBE201077545 and SBE201170578), the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, the 111 Project (No. 111-2-06), the Scientific Research Foundation for Young Scholars of Jiangnan University (No. 2008LQN017), the Scientific Program of Jiangnan University (No. JUSRP11120 and JUSRP111A25), and the Program of the Key Laboratory of Industrial Biotechnology, Ministry of Education, China (No. KLIB-KF200906 and KLIB-KF 201006).

References

1. Amouric A, Brochier-Armanet C, Johnson DB, Bonnefoy V, Hallberg KB (2011) Phylogenetic and genetic variation among Fe(II)-oxidizing acidithiobacilli supports the view that these comprise multiple species with different ferrous iron oxidation pathways. Microbiology 157:111–122
2. Battaglia-Brunet F, Clarens M, Hugues P, Godon JJ, Foucher S, Morin D (2002) Monitoring of a pyrite-oxidising bacterial population using DNA single-strand conformation polymorphism and microscopic techniques. Appl Microbiol Biotechnol 60:206–211
3. Beller HR, Kane SR, Legler TC, Alvarez PJJ (2002) A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. Environ Sci Technol 36:3977–3984
4. Bergamo RF, Novo MTM, Verissimo RV, Paulino LC, Stoppe NC, Sato MIZ, Manfio GP, Prado PI, Garcia O, Ottoboni LMM (2004) Differentiation of *Acidithiobacillus ferrooxidans* and *A. thiooxidans* strains based on 16S–23S rDNA spacer polymorphism analysis. Res Microbiol 155:559–567
5. Bosecker K (1997) Bioleaching: metal solubilization by microorganisms. FEMS Microbiol Rev 20:591–604
6. Chen BW, Liu XY, Liu WY, Wen JK (2009) Application of clone library analysis and real-time PCR for comparison of microbial communities in a low-grade copper sulfide ore bioheap leachate. J Ind Microbiol Biotechnol 36:1409–1416
7. Coram NJ, Rawlings DE (2002) Molecular relationship between two groups of the genus *Leptospirillum* and the finding that *Leptospirillum ferriphilum* sp. nov. dominates South African commercial biooxidation tanks that operate at 40 °C. Appl Environ Microbiol 68:838–845

8. Dave SR, Gupta KH, Tipre DR (2008) Characterization of arsenic resistant and arsenopyrite oxidizing *Acidithiobacillus ferrooxidans* from Hutti gold leachate and effluents. Bioresour Technol 99:7514–7520
9. Demergasso CS, Galleguillos PA, Escudero LV, Zepeda VJ, Castillo D, Casamayor EO (2005) Molecular characterization of microbial populations in a low-grade copper ore bioleaching test heap. Hydrometallurgy 80:241–253
10. Dopson M, Lindström EB (2004) Analysis of community composition during moderately thermophilic bioleaching of pyrite, arsenical pyrite, and chalcopyrite. Microbiol Ecol 48:19–28
11. Dziurla MA, Achouak W, Lam BT, Heulin T, Berthelin J (1998) Enzyme-linked immunofiltration assay to estimate attachment of thiobacilli to pyrite. Appl Environ Microbiol 64:2937–2942
12. Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. Proc Natl Acad Sci USA 103:626–631
13. Frey A, Meckelein B, Externest D, Schmidt MA (2000) A stable and highly sensitive 3,30,5,50-tetramethylbenzidine-based substrate reagent for enzyme-linked immunosorbent assays. J Immunol Methods 233:47–56
14. Herman DJ, Tokunaga TK, Wan JM, Firestone MK (2006) Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl Environ Microbiol 72:6288–6298
15. Hopper DG, Shane J, Straus DC, Kilburn KH, Bolton V, Sutton JS, Guilford FT (2010) Isolation of sulfur reducing and oxidizing bacteria found in contaminated drywall. Int J Mol Sci 11:647–655
16. Hutton JR, Wetmur JG (1975) Activity of endonuclease S1 in denaturing solvents: dimethysulfoxide, dimethylformamide, formamide and formaldehyde. Biochem Biophys Res Commun 66(3):942–948
17. Mahmoud KK, Leduc LG, Ferroni GD (2005) Detection of *Acidithiobacillus ferrooxidans* in acid mine drainage environments using fluorescent in situ hybridization (FISH). J Microbiol Methods 61:33–45
18. Ramírez P, Guiliani N, Valenzuela L, Beard S, Jerez CA (2004) Differential protein expression during growth of *Acidithiobacillus ferrooxidans* on ferrous iron, sulfur compounds, or metal sulfides. Appl Environ Microbiol 70:4491–4498
19. Rawlings DE, Tributsch H, Hansford GS (1999) Reasons why "*Leptospirillum*"-like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores. Microbiology 145:5–13
20. Schrenk MO, Edwards KJ, Goodman RM, Hamers RJ, Banfield JF (1998) Distribution of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*: implications for generation of acid mine drainage. Science 279:1519–1522
21. Smith CJ, Nedwell DB, Dong LF, Osborn AM (2006) Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. Environ Microbiol 8:804–815
22. Stabnikova O, Liu XY, Wang JY, Ivanov V (2006) Quantification of methanogens by fluorescence in situ hybridization with oligonucleotide probe. Appl Microbiol Biotechnol 73:696–702
23. Valdes J, Pedroso I, Quatrini R, Dodson RJ, Tettelin H, Blake R, Eisen JA, Holmes DS (2008) *Acidithiobacillus ferrooxidans* metabolism: from genome sequence to industrial applications. BMC Genomics 9:597
24. Walting HR (2006) The bioleaching of sulphide minerals with emphasis on copper sulphides–a review. Hydrometallurgy 84:81–108
25. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 22:130–138
26. Xie X, Xiao S, He Z, Liu J, Qiu GZ (2007) Microbial populations in acid mineral bioleaching systems of Tong Shankou Copper Mine, China. J Appl Microbiol 103:1227–1238