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DNA Quantification via ICP-MS Using Lanthanide-Labeled Probes and Ligation-Mediated Amplification

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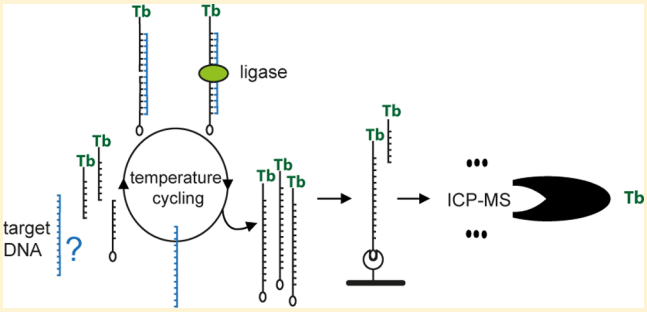
ABSTRACT: The combination of lanthanide-tagged oligonucleotide probes with inductively coupled plasma mass spectrometry (ICP-MS) as the detection technique is a novel labeling and analysis strategy for heterogeneous nucleic acid quantification assays. We describe a hybridization assay based on biotin−streptavidin affinity using lanthanide-labeled reporter probes and biotinylated capture probes. For the basic sandwich type assay, performed in streptavidin-coated microtitration wells, the limit of detection (LOD) was 7.2 fmol of DNA target, corresponding to a final concentration of 6 pM terbium-labeled probes detectable by ICP-MS after elution from the solid support. To improve the sensitivity and sequence specificity of the approach, it was combined with established molecular biological techniques, i.e., elution with a restriction endonuclease and signal and target amplification by the ligase detection reaction (LDR) and ligase chain reaction (LCR), respectively. Initial experiments showed that the enzymes facilitated the discrimination of single-base mismatches within the recognition or ligation site. Furthermore, LCR as a target amplification step resulted in a 6000-fold increase of sensitivity, and finally an LOD of 2.6 amol was achieved with an artificial double-stranded DNA target.



The highly sensitive detection and quantification of specific nucleic acid sequences is needed in biological research, e.g., for the investigation of gene expression levels, and in clinical diagnostics to determine bacterial and viral pathogens as well as genetic disorders. Several methods are well established, namely, the quantitative polymerase chain reaction (PCR), microarray-based techniques, blotting techniques, and others.[1−4] The sequence specificity is obtained by duplex formation of the target nucleic acid sequence with complementary oligonucleotides as probes and primers. Besides nonspecific intercalating agents and label-free techniques, labeled probes serve to detect the nucleic acid hybrids based on fluorescence, chemiluminescence, radioisotopic, and enzyme labels.[5−8]

In this study, we describe a DNA quantification approach using probes labeled with lanthanide chelates in combination with elemental mass spectrometry as the detection technique. Complexes of the macrocyclic chelator 1,4,7,10-tetraazacyclododecane *N*,*N*′,*N*″,*N*‴-tetra acetic acid (DOTA) with trivalent lanthanide ions are known to be thermodynamically stable and kinetically inert. Thus, without the risk to lose or exchange metals, they are well suited as tags for bioanalytical purposes.[9] Applied to protein and peptide analysis, lanthanide tags combined with inductively coupled plasma mass spectrometry (ICP-MS) allow sensitive detection, multiplex analyses and the use of isotopic dilution techniques for absolute quantification.[10,11] In the present work, we adapt this concept to quantitative nucleic acid analysis.

In a recently published proof-of-concept study, Han et al. have shown the multiplexing strength of the approach by simultaneous quantification of 15 DNA targets using 15 different metals.[12] Ornatsky et al. demonstrated the detection of mRNA by in situ hybridization with biotinylated oligonucleotides and analysis via ICP-MS after reaction with lanthanide-labeled streptavidin.[13] Besides the new ICP-MS based techniques, other DNA quantification methods use lanthanide chelates as fluorescence labels; both heterogeneous and homogeneous PCR assays based on time-resolved fluorescence detection have been developed.[14−16] ICP-MS detection is suitable for a heterogeneous assay format only and necessitates the separation of DNA target hybridized with reporter probes from an unbound excess of probes. Instead of using capture probes covalently coupled to the surface of magnetic beads,[12] we performed hybridization of biotinylated capture probes and lanthanide-labeled reporter probes to the DNA target in solution, and then the separation was achieved by biotin−streptavidin affinity.

Different strategies for the synthesis of DOTA−oligonucleotide conjugates have been reported. Oligonucleotides end-modified with a primary amino group were labeled with isothiocyanate-functionalized DOTA derivatives and DOTA amine-reactive ester, whereas thiol-modified oligonucleotides were conjugated with maleimide-functionalized DOTA derivatives.[17−19] An alternative to the labeling in solution is the introduction of DOTA during solid-phase DNA synthesis as an internal or end modification.[20,21]

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Table 1. Sequences and Modifications of Target DNA (D1), Mismatched Target DNA (D2−D5), and Oligonucleotide Probes (D6−D11)[a]

	sequences	modifications
D1	5′-GGTCGGTGCATTAATAAGTGGAATATAATTTTTCAAATCCATATCTACA-3′	
D2	5′-GGTCGGTGCAT**A**AATAAGTGGAATATAATTTTTCAAATCCATATCTACA-3′	
D3	5′-GGTCGGTGCATTAATAAGTGGAATAT**T**ATTTTTCAAATCCATATCTACA-3′	
D4	5′-GGTCGGTGCATTAATAAGTGGAATAT**G**ATTTTTCAAATCCATATCTACA-3′	
D5	5′-GGTCGGTGCATTAATAAGTGGAATAT**C**ATTTTTCAAATCCATATCTACA-3′	
D6	5′-GTAGATATGGATTTG-3′	5′-$C_6H_{12}SH$
D7	5′-ATTAATGCACCGAC-3′	3′-biotin
D8	5′-ATATTCCACTTATTAATGCACC-3′	3′-C_3H_6SH, 5′-phosphate
D9	5′-TGTAGATATGGATTTGAAAAATT-3′	5′-biotin
D10	5′-GTGCATTAATAAGTGGAATATA-3′	5′-$C_6H_{12}SH$
D11	5′-ATTTTTCAAATCCATATCTACA-3′	3′-biotin 5′-phosphate

[a]The mismatched bases are bolded and underlined.

For nucleic acid quantification, ultrahigh sensitivity is needed. The detection of concentrations in the attomolar range is required for many clinical applications. This high sensitivity is obtainable by signal or target amplification.[22] The utilization of DNA ligases for amplification in DNA assays was introduced by Wu et al. and by Barany.[23,24] Two target-specific probes are covalently ligated when both adjacently hybridize to a target DNA sequence. Amplification is achieved by temperature cycling. Upon an increase in temperature, the ligation product dissociates from the target. Upon a decrease in temperature, another pair of free probes can bind and be ligated. The amount of ligation product increases depending on the amount of template DNA and the number of temperature cycles. Amplification proceeds linearly when working with one set of probes (ligase detection reaction, LDR) and exponentially with two complementary probe sets (ligase chain reaction, LCR).[24] LDR applied in (multiplexed) PCR-LDR assays is a powerful technique to discriminate single-base differences.[25,26] Similarly, LCR proved valuable to detect infectious agents and genetic diseases.[27]

EXPERIMENTAL SECTION

Chemicals and Materials. Oligonucleotides were received from Eurofins MWG Biotech in HPLC quality (Eurofins MWG, Ebersberg, Germany). Sequences and modifications are provided in Table 1. Streptavidin-coated microtitration well strips StreptaWell (regular binding capacity) and StreptaWell (high binding capacity) were obtained from Roche (Roche Diagnostics, Mannheim, Germany), and streptavidin-coated microparticles Dynabeads M-280 Streptavidin were obtained from Invitrogen (Invitrogen, Oslo, Norway). 9°N DNA ligase and restriction enzyme *MseI* were from New England BioLabs (NEB, Ipswich, MA). Nitric acid was purchased in optima grade (Fisher Scientific, Loughborough, UK). Water used for all experiments was purified with an USF Elga Purelab Plus system (ELGA, Marlowe, UK).

ICP-MS Detection. ICP-MS measurements were carried out on an Element XR (Thermo Fisher Scientific, Bremen, Germany), equipped with a MicroMist concentric nebulizer (Glass Expansion, West Melbourne Vic., Australia) and a Twinnabar cyclonic spray chamber (Glass Expansion, West Melbourne Vic., Australia). Sample uptake rate by self-aspiration was typically 150 μL min^{-1}. Plasma power was 1300 W, and the nebulizer gas flow was 1.7 L min^{-1}. Ion intensities for ^{159}Tb and ^{209}Bi isotopes were recorded in low resolution mode for 36 s and averaged for further calculations. Standard solutions for external calibration were prepared from multielement lanthanide standard solution in a concentration range 10−1000 ng g^{-1} per element by sequential dilution with 3.5% (v/v) nitric acid. Samples and standard solutions for external calibration were spiked with 100 ng g^{-1} Bi internal standard.

Labeling of Oligonucleotides with Lanthanide Chelate Tags. The maleimide-functionalized DOTA derivative DOTA-Mal-Tb (Figure 1) was prepared as described previously.[28] The thiol-modified oligonucleotides (D6, D8, D10) were treated with 3-fold molar excess of tris(2-carboxyethyl)phosphine (TCEP) for 30 min at 50 °C in 0.1 M phosphate buffer (pH 7.2) to reduce disulfide bonds. Subsequently, DOTA-Mal-Tb was added to the reaction in 5-fold molar excess. The final oligonucleotide concentration in the reaction mixture was 100 μM. The reaction was allowed to proceed overnight at 37 °C. The product was purified by RP-HPLC (HPLC conditions: UV 260 nm, column Phenomenex Gemini-NX, 50 × 4.6 mm, 3 μm, flow 0.8 mL min^{-1}; the solvent system was (A) 95% 25 mM TEAA (pH 5.5), 5% CH_3CN, and (B) 100% CH_3OH; the solvent gradient: 0 −20 min 95−80% A, 20−29 min 80−10% A, 29−31 min 10% A, 31−33 min 10−95% A, 33−35 min 95% A). The purified product was freeze-dried, redissolved in water, and stored at −20 °C until use. Identity was verified by RP-HPLC-ESI-MS analysis (HPLC conditions: column Supelco Discovery BIO Wide Pore C18, 100 × 1 mm, 3 μm, flow 40 μL min^{-1}, the solvent system was (A) 99% 20 mM TEAA (pH 5.5), 1% CH_3OH and (B) 10% 20 mM TEAA (pH 5.5), 90% CH_3OH; the solvent gradient: 0−5 min 90% A, 5−15 min 90−70% A, 15−20 min 70−1% A, 20−30 min 1% A, 30−35 min 1−90% A, 35−45 min 90% A). The Finnigan LTQ FTICR ULTRA mass spectrometer (Thermo Fisher Scientific, Bremen, Germany)

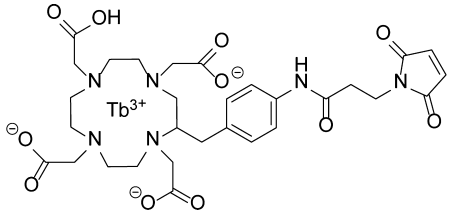

Figure 1. Chemical structure of the labeling reagent DOTA-Mal-Tb.

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was operated in negative ionization mode (m/z 500−4000, electrospray voltage −4.5 kV, capillary temperature 275 °C). D6-DOTA-Mal-Tb calcd m/z 1417.00 $[M\text{-}4H]^{4-}$; found m/z 1417.00; D8-DOTA-Mal-Tb calcd m/z 1917.81 $[M - 4H]^{4-}$; found m/z 1917.78; D10-DOTA-Mal-Tb calcd m/z 1956.59 $[M - 4H]^{4-}$; found m/z 1956.62. For determination of yield, the product was diluted with 3.5% (v/v) HNO_3, spiked with 100 ng g^{-1} Bi internal standard, and analyzed by ICP-MS as described above.

Target DNA. As a simplified model system for assay development, a 49mer DNA oligonucleotide D1 (Table 1) with a sequence belonging to the food-borne pathogen *Listeria monocytogenes*[29] was applied as target DNA in either single-stranded or double-stranded form (ssD1, dsD1). To examine the sequence specificity of the enzyme-based assays, the following targets with single-base mismatches were chosen: D2 with a mismatched base in the *MseI* recognition site and D3, D4, D5, each with a single-base mismatch at the 3′OH side of the LDR ligation site. Double-stranded targets were prepared by mixing the target strand (Table 1) with the correspondent complement strand in equimolar ratio in water, heating to 94 °C for 5 min and slow cooling to room temperature.

Basic Sandwich Hybridization Assay. A specified amount of target DNA ssD1 (50−1000 fmol), 3 pmol of reporter probe D6-DOTA-Mal-Tb, and 2 pmol of capture probe D7 were incubated in a total volume of 60 μL of buffer A (0.1 M phosphate buffer (pH 7.2), 1 M NaCl, 0.01% (w/w) Tween 20) for 3 min at 93 °C, for 10 min at 50 °C, for 30 min at 37 °C, and for 30 min at room temperature. Streptavidin-coated wells (StreptaWell, regular binding capacity) were preincubated with buffer B (0.1 M phosphate buffer (pH 7.4), 1 M NaCl, 0.1% (w/w) BSA, 0.01% (w/w) Tween 20) for 30 min with shaking and rinsed twice with buffer B to block nonspecific binding sites. Subsequently, the hybridization mixture was transferred into the streptavidin-coated cavities and made up to 300 μL with buffer B. Biotinylated hybrids were allowed to bind for 60 min with shaking at room temperature. Wells were rinsed twice with buffer B and once with buffer A to remove unbound reporter probes. To release the immobilized hybrids, the wells were filled with 300 μL of 13% (v/v) nitric acid each and heated to 97 °C for 30 min in a heating block. The hot eluant was transferred to a tube, diluted with 0.75 mL of water, and spiked with 0.15 mL of internal standard solution (800 ng g^{-1} Bi in 3.5% (v/v) HNO_3) to give about 1.2 mL of sample containing 3.5% (v/v) HNO_3 and 100 ng g^{-1} Bi. To correct for losses due to evaporation during heating, the weight of the added internal standard solution and of the total sample solution was used for calculations. Finally, the Tb/Bi response of the samples was monitored by ICP-MS as described above.

Alternative Sandwich Hybridization Assay with Sequence-Specific Elution by Restriction Enzyme. A specified amount of target DNA ssD1 (50−1000 fmol), 3 pmol of reporter probe D8-DOTA-Mal-Tb, and 2 pmol of capture probe D9 were incubated in a total volume of 60 μL of buffer A (0.1 M phosphate buffer (pH 7.4), 1 M NaCl, 0.01% (w/w) Tween 20) for 5 min at 85 °C, for 15 min at 50 °C, for 30 min at 37 °C, and for 30 min at 22 °C, with shaking. Streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, 10 mg beads mL^{-1}, binding capacity for free biotin 650−900 pmol mg^{-1}) were preincubated in buffer B (0.1 M phosphate buffer (pH 7.4), 1 M NaCl, 0.1% (w/w) BSA, 0.01% (w/w) Tween 20) for 30 min with shaking and washed twice in buffer B. Subsequently, 5 μL of beads (10 mg beads mL^{-1}) were given to each hybridization reaction, and the mixtures were made up to 100 μL with buffer B. Biotinylated hybrids were allowed to bind for 90 min with shaking at room temperature. Thereafter, the beads were washed twice in 200 μL buffer B and once with *MseI* reaction buffer (50 mM KOAc, 20 mM Tris-OAc, 10 mM $Mg(OAc)_2$, 1 mM DTT, 0.01% (w/w) BSA, pH 7.9). Immobilized hybridized reporter probes were released from the bead surface by sequence-specific restriction with the enzyme *MseI* (recognition site: 5′-T↓TAA-3′). Therefore, beads were suspended in 100 μL of *MseI* reaction buffer, 5 U (0.5 μL) *MseI* was added, and mixtures were incubated at 37 °C with shaking overnight. Subsequently, the supernatant was transferred to a tube, diluted with 0.1 mL of water, and spiked with 0.2 mL of internal standard solution (200 ng g^{-1} Bi in 7% (v/v) HNO_3) to give 0.4 mL of sample containing 3.5% (v/v) nitric acid and 100 ng g^{-1} Bi. The weight of the added internal standard solution and of the total sample solution was used for calculations. Finally, the Tb/Bi response of the samples was monitored by ICP-MS as described above.

Ligase Detection Reaction. Each reaction contained a specified amount of target DNA (1−20 fmol), either ssD1 or dsD1, 6 pmol of reporter probe D8-DOTA-Mal-Tb, and 4 pmol of capture probe D9 all in a total volume of 10 μL of 9°N DNA Ligase Reaction Buffer (10 mM Tris-HCl, 2.5 mM DTT, 2.5 mM $MgCl_2$, 600 μM ATP, 0.1% Triton X-100, pH 7.5). Four units of 9°N DNA Ligase (1 μL, 1:10 diluted in 9°N DNA Ligase Reaction Buffer) was added, and reactions were incubated in an Eppendorf personal thermal cycler (Eppendorf, Hamburg, Germany). The following temperature cycling conditions were used: an initial denaturation step of 94 °C for 2 min, followed by 60 cycles of 37 °C for 5 min and 94 °C for 1 min. Subsequently, the reaction was terminated by cooling to 4 °C, and the reaction mixture was transferred to streptavidin-coated wells (StreptaWell, high binding capacity, binding capacity 80 pmol of free biotin per well) to separate ligation products from the excess reporter probes. The streptavidin-coated wells with higher binding capacity allow here the application of 4 pmol of capture probes instead of 2 pmol in wells with regular binding capacity. Samples were further processed as described for the basic sandwich hybridization assay.

Ligase Chain Reaction. Each reaction contained a specified amount of target DNA dsD1 (5−60 amol), two complement sets of reporter and capture probe (1.5 pmol of D8-DOTA-Mal-Tb, 1.5 pmol of D10-DOTA-Mal-Tb, 1 pmol of D9, 1 pmol of D11), and 4 U of 9°N DNA Ligase all in a total volume of 10 μL of 9°N DNA Ligase Reaction Buffer. Reactions were kept at 4 °C during preparation. Then the tubes were immediately placed into the preheated thermal cycler and subjected to the following temperature cycling conditions: an initial denaturation step of 94 °C for 2 min, followed by 16 cycles of 37 °C for 5 min and 94 °C for 1 min. The reaction was terminated by rapid cooling to 4 °C. Subsequently, the reaction mixture was transferred to streptavidin-coated wells (StreptaWell, regular binding capacity), and samples were further processed as described for the basic sandwich hybridization assay.

RESULTS AND DISCUSSION

Metal Labeling of Reporter Probes. In a first step, reporter probes had to be tagged with the lanthanide chelates. The readily metalized maleimide-functionalized DOTA derivative DOTA-Mal-Tb (Figure 1), described by Ahrends et

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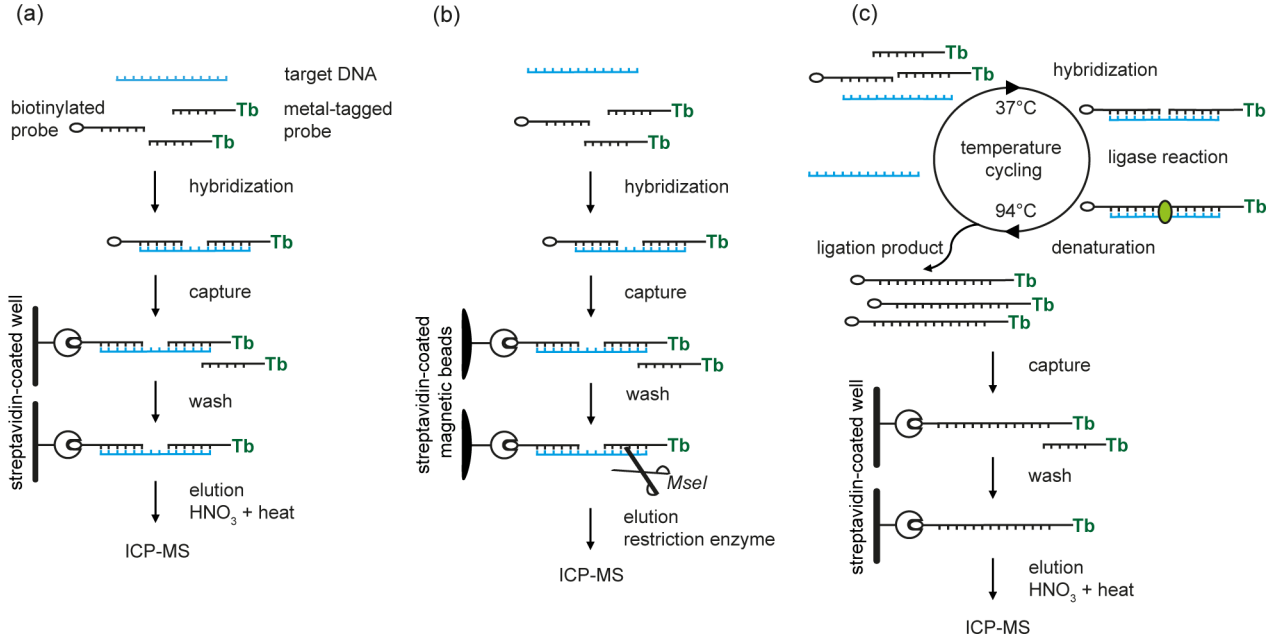
Scheme 1. ICP-MS-Based Sequence-Specific DNA Quantification with Lanthanide-Tagged Probes: (a) Basic Sandwich Hybridization Assay in Streptavidin-Coated Wells, (b) Alternative Sandwich Hybridization Assay with Streptavidin-Coated Magnetic Beads and Elution by Restriction Enzyme, (c) Assay with Ligation-Mediated Amplification



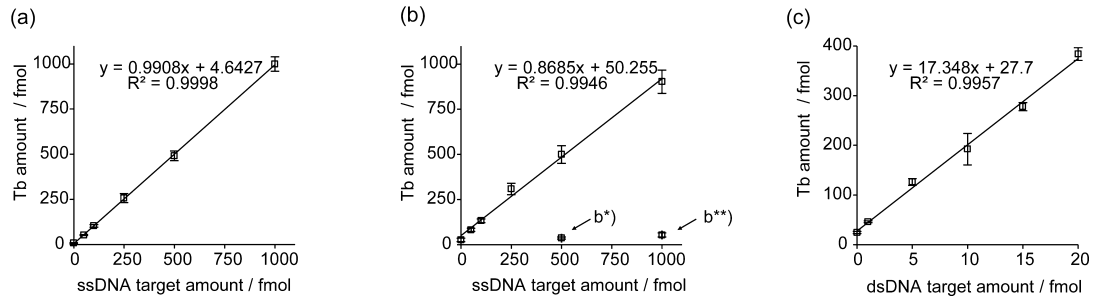


Figure 2. Determined amount of Tb as a function of target DNA amount: (a) basic sandwich hybridization assay with ssDNA target; (b) alternative sandwich hybridization assay with ssDNA target and elution by restriction enzyme *MseI*, including b* and b** as controls without *MseI* and with 500 fmol and 1000 fmol of ssDNA target, respectively; (c) ligase detection reaction-based assay with dsDNA target, 60 cycles resulted in a 17-fold signal amplification. Error bars represent the standard deviation of triplicates.

al.,[10] was attached to thiol-modified oligonucleotides in solution via thiol−ether bond formation. A 5-fold excess of the reagent ensured complete labeling of the 15mer and 22mer oligonucleotides. The metal-labeled reporter probes were purified by HPLC, identity and purity was verified by HPLC-MS, and finally their concentration was determined by ICP-MS analysis.

Basic Sandwich Hybridization Assay. For the development of the approach, a synthetic 49mer oligonucleotide was chosen as DNA target in both single- and double-stranded form (ssDNA and dsDNA target). The assays workflows are illustrated in Scheme 1. In the basic sandwich hybridization assay (Scheme 1a), streptavidin-coated microtitration wells were employed as the solid support. The formation, capture, and release of the hybrids was quantitative with the ssDNA target. The determined amount of terbium was almost equal to the amount of DNA target input. To achieve complete capture, the amount of biotinylated probes had to be restricted to 2 pmol per well. The binding capacity for the free biotin was 20 pmol per well (supplier's information). In terms of complete liberation from the solid support and low background, the elution with 13% (v/v) nitric acid and heat proved to be superior to elution with water and heat or elution with 3.5% (v/v) nitric acid, 0.1% (w/v) sodium dodecyl sulfate, and heat (data not shown). The eluted sample was diluted, spiked with bismuth as internal standard, and analyzed by ICP-MS. Figure 2a displays an assay calibration curve; the linear range was about 10−1000 fmol of ssDNA target. The limit of detection (LOD) is set by the assay background. Assay blank values determined with reporter probes were similar to values without reporter probes; thus, the washing procedure was sufficient. The assay blank value with probes was 7.6 ± 2.2 fmol of Tb (mean of triplicates ± standard deviation (SD)), corresponding to a final concentration of 6.4 pM Tb or 1.0 ng g^{-1} Tb. Thus, an LOD of 7.2 fmol of ssDNA target was calculated (defined as 3.3 × SD of blank/slope of calibration curve).

Sandwich Hybridization Assay with Elution by Restriction Enzyme. We also conducted experiments with streptavidin-coated magnetic beads as the solid support. As the elution with nitric acid is not compatible with this support, we employed the restriction enzyme *MseI* to cut the double-stranded DNA hybrid and, thereby, liberate the metal label

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(Scheme 1b). An assay calibration curve is shown in Figure 2b. The assay blank value with probes was 26.2 ± 9.2 fmol of Tb (n = 3). The LOD was calculated to 34.9 fmol of target DNA. Control reactions with probes and target but without restriction enzyme showed that the unspecific release was low (Figure 2b). The main advantage is the additional sequence specificity. The enzyme recognition site is formed by the reporter probe and the DNA target. Therefore, mismatched targets that are not harboring the recognition site are discriminated. The selectivity of this enzymatic elution was proven by a mismatched target with a single-base substitution in the recognition site. As shown in Figure 3, no elution occurred. In contrast, when sequence

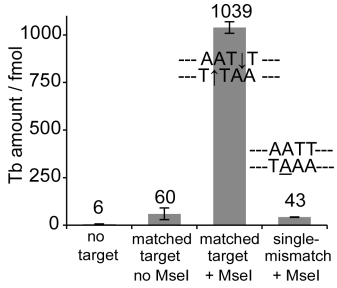

Figure 3. Specificity of the sandwich hybridization assay with *MseI* restriction enzyme elution. Left to right: control without DNA target; control 1000 fmol of ssDNA target without *MseI* (unspecific release); 1000 fmol of ssDNA target with *MseI*; 1000 fmol of single-base mismatch target with *MseI*. Error bars represent the standard deviation of triplicates.

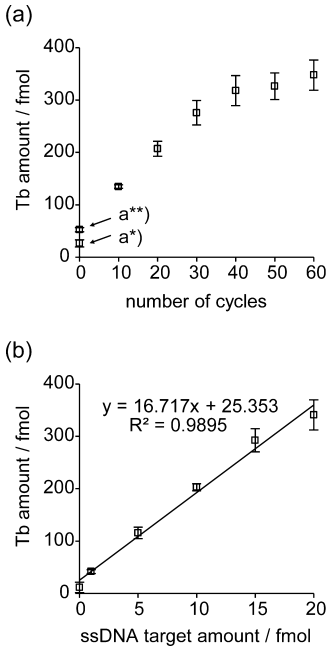

Figure 4. Determined amount of Tb in LDR assays: (a) amount of Tb as a function of temperature cycles, replicates of 20 fmol of ssDNA target each, including a* as the assay blank value performed with probes and a** as the assay with 20 fmol of ssDNA target without temperature cycling; (b) amount of Tb as a function of ssDNA target amount, 60 cycles resulted in a 17-fold signal amplification. Error bars represent the standard deviation of triplicates.

specificity is based solely on duplex formation, a precise optimization of temperature, probe sequence, and salt concentration is required to minimize the detection of similar sequences. Another advantage of this assay is the possibility to increase the concentration range by adding more magnetic beads. However, compared to the elution with nitric acid, the multiplexing capabilities are restricted to targets cleavable by the same enzyme or by different enzymes active under the same conditions.

Amplification by Preceding Ligase Reactions. The use of ligase reactions (LDR, LCR) to enhance the assay sensitivity was investigated. The ATP-dependent heat-stable 9°N-DNA-ligase (from *Thermococccus sp.* (strain 9°N)) was employed to ligate lanthanide-labeled reporter probes and biotinylated capture probes (Scheme 1c). The enzyme catalyzed the formation of a phosphodiester bond between the 3′-OH and 5′-phosphate groups to join the two probes when both bound adjacently to a target DNA molecule. The process was repeated by temperature cycling, resulting in an accumulation of ligation products. Upon completion of the amplification process, ligation products were isolated in streptavidin-coated wells, eluted with nitric acid, and quantified via ICP-MS. Reaction conditions (enzyme amount and cycling parameter) were initially optimized for LDR, i.e., ligase reaction with one probe-set. To study the progress of target-templated ligation, replicate reactions with 20 fmol of ssDNA target each were prepared, run simultaneously, and stopped after 0, 10, 20, 30, 40, 50, and 60 cycles. The amplification progress was virtually linear during the first 30 cycles (Figure 4a). After 60 cycles, a 17-fold amplification was gained, whereas no amplification was observed without target. While in the basic sandwich hybridization assay recovery was low with dsDNA target (data not shown), assays with preceding LDR worked equally well with both ssDNA and dsDNA targets (Figures 2c and 4b). The assay blank value with probes was 24.6 ± 2.2 fmol of Tb (n = 3) (Figure 2c), resulting in an LOD of 0.4 fmol of dsDNA target. The linear range was about 1−20 fmol of DNA target. The upper limit was given by the capacity of the streptavidin-coated wells and the requirement to work with an excess of probes (in our setup at least 20-fold), as probes have to outcompete the emerging ligation product. As the amplification yield of LDR was limited, we extended our investigations to LCR by adding a second probe set complement to the first one and, thereby, allowed the formed ligation products to serve as templates for subsequent cycles. An exponential progress of amplification was observed when running replicate reactions with 60 amol of dsDNA target each for 0, 10, 12, 14, and 16 cycles (Figure 5a). An assay calibration curve in the range of 5−60 amol of dsDNA target showed that after 16 cycles a 6000-fold amplification was reached (Figure 5b). The assay blank value (with probes and ligase) was 14.7 ± 5.2 fmol of Tb (n = 4), resulting in an LOD of 2.6 amol of dsDNA target, i.e., 1.6×10^6 copies.

Sequence Specificity of the Ligase Detection Reaction. In addition to the amplification effect, ligase reactions might enable a facile distinction of single-base mismatches at the ligation site, an advantage over the PCR techniques. To initially evaluate the ligase reaction specificity, LDR experiments were performed to test four DNA targets with the probe set D8/D9. Besides the dsDNA target, fully complementary to the probe set, three mismatched dsDNA targets with a single-base substitution at the 3′-OH side of the nick were assayed. Compared to the matched target base pair T:A, no amplification was observed with the single T:T and T:G mismatched DNA, whereas significant ligation occurred in the T:C mismatch case (Figure 6). The experiments summarized in

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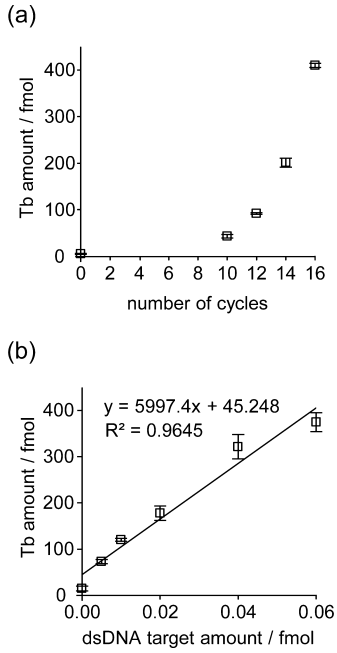

Figure 5. Determined amount of Tb in LCR assays: (a) amount of Tb as a function of temperature cycles, replicates of 0.06 fmol of dsDNA target each; (b) amount of Tb as a function of dsDNA target amount, 16 cycles resulted in an 6000-fold amplification. Error bars represent the standard deviation of triplicates.

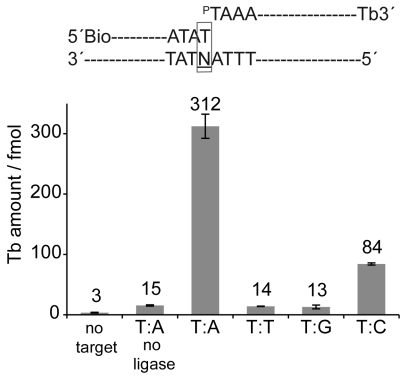

Figure 6. Specificity of the LDR assay toward single-base mismatches at the 3′-OH side of the nick using 60 cycles and 20 fmol of dsDNA target each. Left to right: control without target; control matched dsDNA target T:A without ligase; matched dsDNA target T:A with ligase; single-base mismatched dsDNA targets with ligase T:T, T:G, T:C. Error bars represent the standard deviation of quadruplicates.

Figure 6 were performed, applying a ligation temperature of 37 °C. Trials with increased ligation temperature (45 °C, 55 °C, 60 °C) resulted unexpectedly in a higher extent of mismatch ligation (data not shown). The sequence specificity of ligase reactions depends on the chosen enzyme and might be influenced by the sequence surrounding the nick, the ligation temperature, and the buffer composition.[30−34] When considering the assay selectivity, it must be noted that the capture step as currently performed does not distinguish between the ligation product and mismatched but stable hybrids containing nontarget DNA and reporter and capture probes. Both are immobilized and detected. This will not be crucial for LCR, because the ligation product is formed in high excess compared to the native nontarget DNA. In the case of LDR, an additional step might be required to remove DNA noncovalently bound to the biotinylated probes, for instance, by performing the capture step at elevated temperature, by denaturation with diluted NaOH, or by pretreatment with an exonuclease.[35] Both the specificity of the ligase reaction and the specificity of the capture step have to be further examined and optimized when changing over from the simplified model system to biological sample DNA.

CONCLUSIONS

We report our first results employing lanthanide-chelate-labeled oligonucleotide probes in combination with ICP-MS detection as a novel labeling and analysis strategy for heterogeneous nucleic acid quantification assays. The lanthanide tag is small, stable, and heat-resistant; it is not prone to nonspecific binding, and the lanthanides are rare in biological samples. The high multiplexing capacity, an important advantage of the approach compared to common fluorescence labels, was most recently demonstrated.[12] In the present work, we combined the technique with established molecular biological methods, namely, restriction enzyme digestion and the ligase reaction, and improved both the selectivity, distinguishing single nucleotide differences, and the sensitivity. The LODs were 7.2 fmol of ssDNA target for the basic sandwich hybridization assay and down to 2.6 amol of dsDNA target with the preceding LCR. Thus, the combination of the novel label with enzyme-based amplification methods allows us to reach the sensitivity range required for DNA quantification at biologically relevant levels. In the future, the LOD might be further improved by increasing the number of LCR cycles and by applying tags containing more than one lanthanide ion. Despite the ICP-MS main drawback of being an expensive technique and rather rare in clinical and biological research laboratories, the lanthanide-tagged nucleic acid detection approach might further evolve in combination with other amplification methods or with an alternative instrumental setup, for example, laser ablation ICP-MS from a microarray chip or capillary electrophoresis ICP-MS.

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Notes

The authors declare no competing financial interest.

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