DNA Detection

DOI: 10.1002/anie.201001529

Ultrasensitive DNA Detection by Cascade Enzymatic Signal Amplification Based on Afu Flap Endonuclease Coupled with Nicking Endonuclease**

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Detection of specific sequences in target DNA at an ultralow level is critical to clinical diagnosis. Many approaches based on various principles have thus been developed, such as the polymerase chain reaction (PCR),[1] nucleic acid sequence-based amplification (NASBA),[2] rolling-cycle amplification (RCA),[3] and loop-mediated isothermal amplification (LAMP).[4] However, these methods are based on template replication, which increases the risk of cross-contamination from amplicons, so false-positive results frequently occur. Amplification of target-DNA-specific signals instead of target DNA itself is an effective way to solve this problem. Up to now there have been several strategies based on signal amplification to sensitively detect DNA molecules, including branched DNA (bDNA),[5,6] invasive signal amplification,[7,8] and nicking endonuclease signal amplification (NESA).[9–11] NESA is a newly developed method that is promising for sensitive DNA detection. In NESA, probes binding to a target containing a nicking endonuclease (NEase) recognition sequence are nicked by the NEase, which cleaves one strand in a DNA duplex, and nicked probe species dissociate from the target followed by the annealing of intact probes; 120 min of this process leads to the generation of more than 1000 nicked probes from one target. However, the requirement of the NEase recognition site in a target sequence greatly limits the use of NESA in various applications.

On the other hand, the sensitivity of NESA is much lower than that of the PCR; thus an additional amplification step is required for detecting target DNAs at an ultralow level. An effort to allow NESA to sensitively detect any target sequence of interest was made by employing a padlock probe to capture a target DNA followed by RCA. Although 0.1 pM target DNA was successfully detected,[10] the RCA process is not a "real" signal amplification because RCA amplicons, which keep intact after NESA, contain replicated primary-target sequences complementary to the padlock probe; hence the risk of cross-contamination still exists. To achieve an ultrasensitive "real" signal amplification, we used *Archaeoglobus fulgidus* (Afu) flap endonuclease-based invasive signal amplification[12,13] to generate amplified templates for NESA. Unlike NESA, invasive signal amplification does not rely on specific recognition sequences in a target but on a specific structure formed by the specific binding of an upstream probe and a downstream probe to a target DNA; so any target sequence can be detected. To bridge NESA and invasive signal amplification, a molecular-beacon-based ligation reaction[14] was employed.

The principle of the proposed cascade enzymatic signal amplification (CESA) is illustrated in Figure 1, and includes three steps: invasive signal amplification, flap ligation, and NESA. Firstly, a downstream probe and an upstream probe specific to the target of interest are designed. If the upstream probe–target DNA duplex overlaps the downstream probe–target DNA duplex by one base pair, the 5′ flap of the downstream probe is cleaved by Afu flap endonuclease. Because the cleavage reaction is carried out at a temperature around the melting temperature (T_m) of the downstream probe, the cleaved downstream probe can rapidly dissociate from the target; as it has a much higher concentration than target DNAs, the intact downstream probe predominates over the cleaved probe in annealing to the target DNAs. Flap accumulation is thus initiated by the cycle of cleavage–dissociation–annealing. At this stage, several thousands of flaps (signals) can be produced from one target molecule.[8] As there is no requirement for an endonuclease-specific recognition sequence in a target DNA, any target can be detected.

Secondly, a molecular beacon (MB) hybridizing with a 5′-phosphorylated oligonucleotide (P-oligo) in the loop of MB is used to capture the amplified flaps; a ligation reaction occurs when the flaps hybridize to the MB at a position adjacent to the 5′ end of the P-oligo. The ligated flap opens the stem of the MB, thus forming the full recognition site of the NEase (e.g., Nb.BsmI). Thirdly, a NEase is added to nick the MB strand in a MB-ligated flap duplex. After the nicking reaction at 60 °C, the cleaved MB pieces spontaneously dissociate from the ligated flap strand, and the fluorescently labeled oligonucleotides produce signals. Subsequently, the ligated flap strand will be captured by another intact MB, which generates

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[**] This work was supported by the National Natural Science Foundation of China (Grant 20975113) and the National Key Science & Technology Special Project (2008zxj09014-012).

Afu = *Archaeoglobus fulgidus*.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201001529.

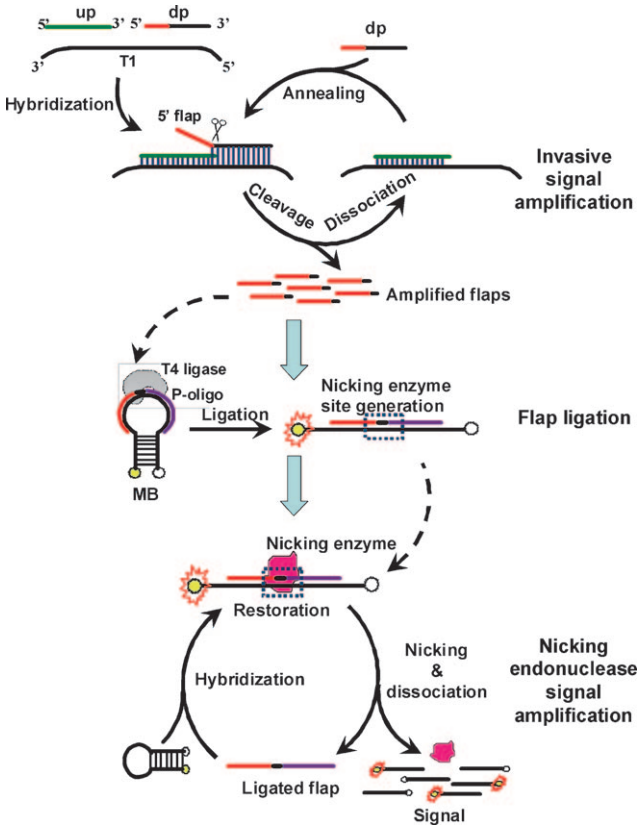

Figure 1. Principle of CESA for target DNA detection. There are three steps: invasive signal amplification by AFU endonuclease to generate amplified flaps, flap ligation by T4 ligase to form a nicking site, and nicking reaction by NEase to produce amplified signals. up = upstream probe, dp = downstream probe, T1 = target of interest.

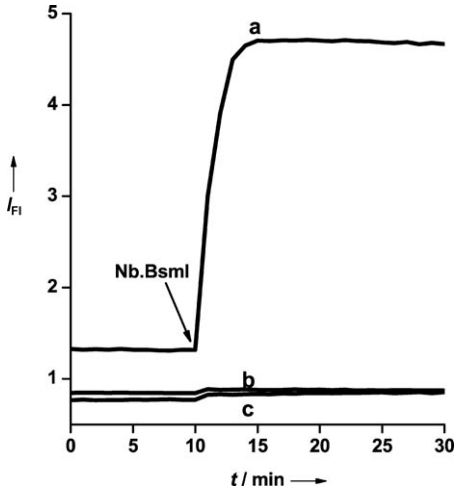

Figure 2. Time courses of NESA in the presence (curve a) or absence (curves b and c) of flap ligation. I_{FI} = fluorescence intensity. Ligation was performed by adding T4 ligase to a mixture having two ready-to-ligate probes hybridized with the loop of the MB. After ligation, NESA of ligated flaps starts by adding Nb.BsmI (indicated by the arrow). Curves b and c are for NESA controls without a recognition site (no T4 ligase) and without a target sequence (no flaps), respectively.

the cycle of nicking–dissociation–hybridization. In this step, ligated flaps are templates of the nicking reaction, and each ligated flap leads to around 1000 fluorescently labeled oligonucleotides. Therefore, the cascade signal amplification by the two kinds of endonucleases results in a significant increase in the sensitivity of DNA detection; theoretically, one target DNA molecule can produce at least one million cleaved MBs by CESA.

One of the key steps in CESA is the flap ligation, which is essential to couple invasive signal amplification with NESA. To investigate whether or not the opening of the stems of MBs depends on the ligation reaction, incubation experiments with two ready-to-ligate probes (flap and P-oligo) hybridized with the loop of the MB in the presence or absence of T4 ligase were carried out. As shown in Figure 2 (0–10 min time course), a signal (curve a) from the restoration of the MB occurs in the presence of T4 ligase, but no signal (curve b) was observed in the absence of T4 ligase, thus indicating that the opening of the MB is specific to the ligation reaction. To further validate the specificity of NEase, Nb.BsmI was added to the above experiments (with and without T4 ligase) individually. It was found that signal amplification from the nicking reaction only took place when the MB hybridized with the ligated probes (curve a in Figure 2, > 10 min time course). Therefore, NEase is specific to the target sequence, and the cleavage by NEase will not happen if no ligase (curve b in Figure 2, > 10 min) or no flaps (curve c in Figure 2, > 10 min) exist in the ligation reaction.

As the flaps produced by invasive signal amplification are subjected to the subsequent ligation reaction, competition in hybridization to the MB between the higher concentration of intact downstream probe (200 nM) and the lower concentration of cleaved flaps (< 1 nM) could be an issue to the amplification efficiency. To overcome this issue, a large amount of MB (800 nM) was used; thus, flaps can be fully captured by the excess MB.

In CESA, three enzyme reactions are included and each enzyme has a specific buffer condition, including invasive buffer (10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.05% Tween-20, 0.05% Nonidet P40, 7.5 mM $MgCl_2$, pH 7.5), ligation buffer (66 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM adenosine 5′-triphosphate (ATP), 7.5% polyethylene glycol (PEG) 6000, pH 7.6), and nicking buffer (0.1M NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). Therefore, the effects of the carryover components from invasive buffer on ligation efficiency, as well as the effects of the carryover components from both the invasive buffer and ligation buffer on the nicking reaction, should be investigated. As shown in Figure S1 in the Supporting Information, the ligation efficiency was little influenced by the carryover components from the invasive reaction buffer, but the carryover components (Tween-20, DTT, PEG 6000, and Nonidet P40) significantly increased the efficiency of the nicking reaction (see Figures S2 and S3 in the Supporting Information). To look for an optimized buffer condition for the nicking reaction, each of the four carryover components was spiked into CESA buffer individually, and the nicking efficiency was not further increased (see Figure S4 in the Supporting Information), which suggests that the CESA buffer mixture is an optimized condition for the nicking reaction. Further study indicated

that the detection limit of the nicking reaction by CESA buffer is superior to that by the nicking buffer from the NEase supplier (see Figure S5 in the Supporting Information).

To examine the sensitivity of CESA, we performed an invasive amplification reaction with various amounts of template (T1): 1 fmol, 100 amol, 10 amol, 1 amol, 0.1 amol, and 0 amol. After 2 h of incubation, a ligation reaction was carried out by adding ligation-ready components in the same tube at room temperature; then NEase (50 U of Nb.BsmI) was added for the nicking reaction in a final volume of 100 μL. Figure 3 shows the time courses of CESA with different target concentrations. It was observed that the fluorescence intensity increased almost linearly with time until a plateau was reached. The detection limit of CESA was determined as 1 fM, which is nearly two orders of magnitude higher than that of NESA coupled with RCA assay (100 fM), and more than four orders of magnitude higher than that of basic NESA (10 pM).[10] Therefore, the combination of Afu flap endonuclease-based invasive amplification with NESA not only enables the detection of any target sequences, but also significantly increases the sensitivity of NEase-based amplification. We believe that the ultrahigh sensitivity of CESA is from the first step of invasive signal amplification, which presents an amplification of more than 10000-fold (see Figure S6 in the Supporting Information) even if the amount of target DNA is as low as 0.1 amol (1 fM in 100 μL; see Figure S7 in the Supporting Information).

Unlike real-time PCR, quantification of target DNA by CESA seems difficult since two steps of signal amplification are coupled. A simple method is to perform CESA on targets with serially diluted concentrations, and the comparison of time courses between the known and the unknown could give a rough concentration of a sample. An accurate way for target quantification is the use of standard curves. As the steady-state reaction rate of either invasive amplification or NESA is proportional to the target concentration,[10,15] reaction rates can be employed to quantify target DNA. If the initial concentration of the target is $[C]_0$, and the cycling cleavage rate (the number of flaps produced from a target molecule per unit of time) in invasive reaction is defined as α, the initial cleavage rate of the reaction can be expressed as $d[C_{flap}]/dt = \alpha[C]_0$.[15] In the case of ultrasensitive detection, $[C]_0$ is much lower than the concentration of the downstream probe, so no plateau of the invasive reaction will be reached at a given time period (usually < 2 h); the final concentration of cleaved flaps can thus be expressed as $[C_{flap}] = \alpha[C]_0 T$ after reaction time T. Provided that the efficiency of the ligation reaction is β, which is constant under fixed ligation conditions, the concentration of ligated flaps subjected to the nicking reaction should be $\beta[C_{flap}]$. According to the kinetics of NESA,[10] the initial rate of NESA (V_0) can be expressed as $V_0 = \alpha'\beta[C_{flap}] = \alpha'\beta\alpha[C]_0 T$, where α' is a pseudo first-order rate constant of the nicking reaction;[10] V_0 is hence proportional to $[C]_0$ (the initial concentration of the target). V_0 at various target concentrations can be measured from the corresponding time courses in CESA, and a standard curve can thus be readily obtained. Based on the CESA data at 10 pM, 1 pM, 100 fM, 10 fM, and 1 fM target DNA (T1), the relationship between V_0 and $[C]_0$ was plotted in Figure S8 in the Supporting Information, which indicates an obvious linear relationship ($R^2 = 0.9843$).

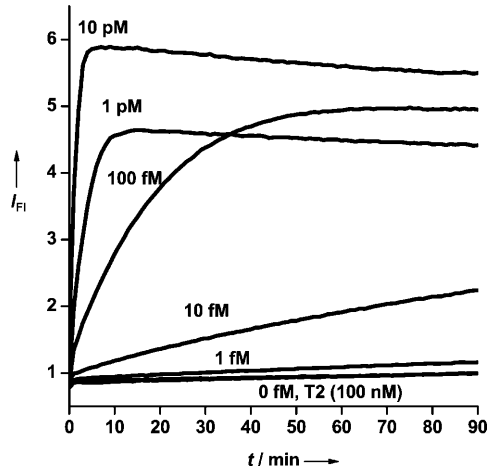

Figure 3. Time courses of CESA of target T1 (complementary to the invasive probes) at the concentrations indicated and target T2 (non-complementary to the invasive probes) at 100 nM.

In CESA, flap ligation and NESA are independent of target sequence, so the specificity of CESA only depends on invasive reaction. As Afu flap endonuclease only recognizes the "invaded structure", no cleavage occurs if the upstream and downstream probes do not hybridize with the target. Results of CESA in the detection of the target (T2) DNA noncomplementary to the invasive probes show the same signal intensity as a blank control (see Figure 3). To further check the specificity of CESA, a series of artificial targets having one base mutated at different locations (a–f in Figure 4) were detected by CESA, and showed that the initial reaction rates of the mutation bases a–d dramatically decreased in comparison with the no-mutation target (P), very close to the blank control (N). Thus, the specificity of CESA is much higher when the invasive structure is not formed as a result of mutation (bases a and d), or the downstream probe–target duplex has a noncomplementary base (bases b and c), while the specificity is not good when the upstream probe–target duplex has a noncomplementary base (bases e and f). The reason is believed to be the higher T_m of the upstream probe–target duplex than that of the downstream probe–target duplex, and a noncomplementary base in the downstream probe–target duplex significantly affects the stability of the duplex. This feature will benefit the design of CESA for genotyping.

In conclusion, we have successfully achieved CESA by coupling Afu flap endonuclease-based amplification with NEase-based amplification. The sensitivity is as high as 1 fM DNA, approaching that of the PCR. Different from the conventional NESA, CESA does not need the target of interest to have a NEase recognition sequence, so any target can be detected. On the other hand, CESA is cost-effective as reagents for flap ligation and nicking amplification are universal for any DNA sequence detection. In the case of a new target DNA, only two dye-free target-specific oligos (downstream and upstream probes) need to be synthesized before detection. It should be mentioned that the target-

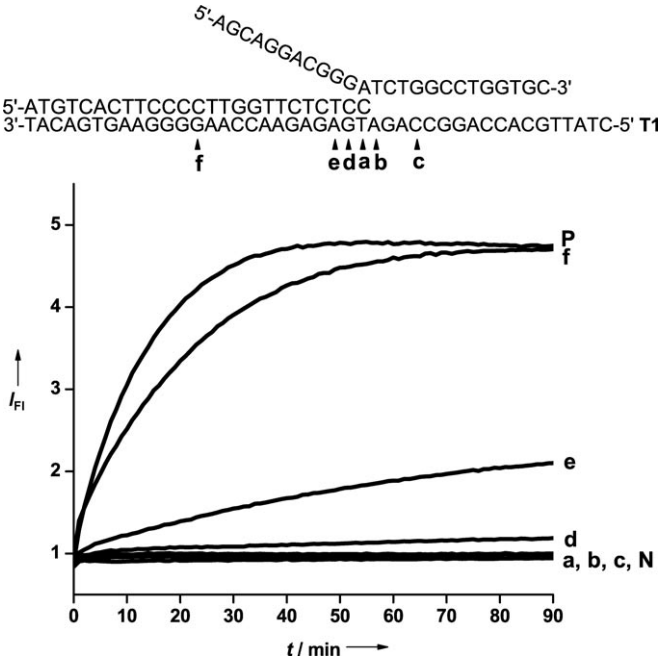

Figure 4. Time courses of CESA of targets artificially mutated at different locations (a–f). All mutations are made by substituting the original base with its complementary one, and are labeled below the sequence. The concentration of all targets is 1 pM. P and N represent a target without any mutation and a control without any target, respectively.

specific sequence in a downstream probe should be designed at a melting temperature much closer to the reaction temperature, because the amplification efficiency of the invasive reaction is based upon the association and dissociation of the downstream probe–target DNA duplex. Moreover, the operation of CESA is quite simple because we only need to open the tube for the addition of reagents at two steps: flap ligation and nicking reaction. The use of a liquid-handling automated system can further simplify the operation. Although CESA is not a closed-tube detection system, there is no risk of amplicon cross-contamination because CESA is based on "real" signal amplification. Thus, the application of CESA to the in situ rapid diagnosis of infectious disease out of a regular laboratory is possible. Because of the constant-temperature reaction, inexpensive CESA instrumentation can be readily achieved by employing a simple heater with a temperature controller. Although the sensitivity of CESA is as low as 1 fM, it is still challenging to detect a target with an amount below 0.1 amol (in 100 μL); thus, the sensitivity should be further improved. Coupling serial invasive signal amplification[7] with NESA could be an effective way for the increase of CESA sensitivity.

Experimental Section

Firstly, invasive signal amplification was performed by adding different amounts (1000, 100, 10, 1, 0.1, and 0 amol) of 43-nucleotide (nt) target DNA (5′-CTA TTG CAC CAG GCC AGA TGA GAG AAC CAA GGG GAA GTG ACA T-3′, T1) to a reaction buffer containing 10 mM MOPS (pH 7.5), 0.05% Tween-20, 0.05% Nonidet P40, 0.1 μM upstream probe (5′-ATG TCA CTT CCC CTT GGT TCT CTC C-3′, up, T_m: 68 °C), 1 μM downstream probe (5′-AGC AGG ACG GGA TCT GGC CTG GTG C-3′, dp, T_m: 63.1 °C), and 7.5 mM $MgCl_2$. After reaction at 95 °C for 5 min, Afu flap endonuclease (71 ng; prepared in our laboratory, see Figures S9–S11 in the Supporting Information for details) was added to the above mixture (total volume 10 μL) and the mixture was incubated at 63 °C for 2 h. For the specificity detection, a 43-nt oligonucleotide 2 (5′-TAC GAG ACC TCC CGG GGC ACT CGC AAG CAC CCT ATC AGG CAG T-3′, T2, 10 pmol) was used.

After flap amplification, ligation mixture (39.5 μL) containing 2 × ligation buffer (25 μL, 132 mM Tris–HCl, 20 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 15% PEG 6000, pH 7.6, New England Biolabs, USA), P-oligo (4.8 μL, 10 μM, 5′-P-ATGCACTCATCTA-3′;), MB (4.0 μL, 10 μM, 5′-FAM-CGC ACG CTA GAT GAG TGC ATT CCC GTC CTG CTG CGT GCG-dabcyl-3′, TaKaRa, China), and water (5.7 μL) was added, followed by incubation at 45 °C for 5 min. T4 ligase (0.5 μL, 17.5 U μL^{-1}, TaKaRa, China) was added at 26 °C, and then the mixture was kept at 26 °C for 15 min.

To perform NESA, nicking mixture (50 μL, 0.2 M NaCl, 20 mM Tris–HCl, 20 mM $MgCl_2$, 2 mM DTT, Nb.BsmI 50 U, pH 7.9, New England Biolabs, USA) was added to the ligated products in a volume of 100 μL. Readout was carried out with an MJ Opticon 2 continuous fluorescence detector (MJ Research Corp., USA).

Received: March 14, 2010
Revised: September 3, 2010
Published online: November 24, 2010

Keywords: DNA cleavage · endonucleases · molecular beacons · sequence determination · signal amplification

[1] K. B. Mullis, F. A. Faloona, *Methods Enzymol.* **1987**, *155*, 335.
[2] J. Compton, *Nature* **1991**, *350*, 91.
[3] P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas, D. C. Ward, *Nat. Genet.* **1998**, *19*, 225.
[4] T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, T. Hase, *Nucleic Acids Res.* **2000**, *28*, 6e3.
[5] M. L. Collins, B. Irvine, D. Tyner, E. Fine, C. Zayati, C. Chang, T. Horn, D. Ahle, J. Detmer, L. P. Shen, J. Kolberg, S. Bushnell, M. S. Urdea, D. D. Ho, *Nucleic Acids Res.* **1997**, *25*, 2979.
[6] S. Capaldi, R. C. Getts, S. D. Jayasena, *Nucleic Acids Res.* **2000**, *28*, 21e.
[7] J. G. Hall, P. S. Eis, S. M. Law, L. P. Reynaldo, J. R. Prudent, D. J. Marshall, H. T. Allawi, A. L. Mast, J. E. Dahlberg, R. W. Kwiatkowski, M. de Arruda, B. P. Neri, V. I. Lyamichev, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8272.
[8] V. Lyamichev, A. L. Mast, J. G. Hall, J. R. Prudent, M. W. Kaiser, T. Takova, R. W. Kwiatkowski, T. J. Sander, M. de Arruda, D. A. Arco, B. P. Neri, M. A. Brow, *Nat. Biotechnol.* **1999**, *17*, 292.
[9] T. Kiesling, K. Cox, E. A. Davidson, K. Dretchen, G. Grater, S. Hibbard, R. S. Lasken, J. Leshin, E. Skowronski, M. Danielsen, *Nucleic Acids Res.* **2007**, *35*, e117.
[10] J. J. Li, Y. Chu, B. Y. Lee, X. S. Xie, *Nucleic Acids Res.* **2008**, *36*, e36.
[11] W. Xu, X. Xue, T. Li, H. Zeng, X. Liu, *Angew. Chem.* **2009**, *121*, 6981; *Angew. Chem. Int. Ed.* **2009**, *48*, 6849.
[12] M. W. Kaiser, N. Lyamicheva, W. Ma, C. Miller, B. Neri, L. Fors, V. I. Lyamichev, *J. Biol. Chem.* **1999**, *274*, 21387.
[13] V. Lyamichev, M. A. Brow, J. E. Dahlberg, *Science* **1993**, *260*, 778.
[14] Z. Tang, K. Wang, W. Tan, J. Li, L. Liu, Q. Guo, X. Meng, C. Ma, S. Huang, *Nucleic Acids Res.* **2003**, *31*, 148e.
[15] V. I. Lyamichev, M. W. Kaiser, N. E. Lyamicheva, A. V. Vologodskii, J. G. Hall, W. P. Ma, H. T. Allawi, B. P. Neri, *Biochemistry* **2000**, *39*, 9523.

