Advancing DNA Steganography with Incorporation of Randomness

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Supporting Information
1. Supporting figures

Figure S1. Sanger sequencing chromatogram of i+d-DNA at the ratio of 1:1 (a) and 1:10 (b).
**Figure S2.** (a) i-DNA and d-DNA was mixed in ten different ratios in the total amount of 1 pmol. (b) qPCR amplification curves of all ratios before (red) and after (key-2) treatment. (c) ΔCt (Ct of sample after key-2 treatment - Ct of sample before Key-2 treatment) values of DNA in ten ratios (A-K).
Figure S3. qPCR melting curves of DNA samples (A-K). Red: before key-2 treatment. Blank: after key-2 treatment.
Figure S4. Comparison of key-2 treated i+d-DNA with untreated i+d-DNA at ratio of 1:2, 1:3, and 1:4 via qPCR amplification curve (a) and melting curve (b and c). Sanger sequencing chromatogram of i+d-DNA at the ratio of 1:1000 (d), 1:500 (e), and 1:200 (f) after key-2 treatment.
Figure S5. Evaluation of the method on different amount of template DNA. (a) 0.1 pmol, (b) 0.01 pmol, (c) 1 fmol, (d) 0.1 fmol. Red: DNA before key-2 treatment. Black: DNA after key-2 treatment. Blue: Non-templated control with the same primer concentration (blue). (e) ΔCt of DNAs of different amount before and after key-2 treatment. (f) Sanger sequencing chromatogram of i-DNA retrieved from 1 fmol i-DNA: d-DNA (1:100) after key-2 and key-1 treatment.
Figure S6. Four i-DNAs and corresponding d-DNAs were mixed to form 0.1 pmol. For each i-DNA, d-DNA: i-DNA=1:100. (a-d) qPCR measurement before (red) and after (black) key-2 treatment (EcoRV and BamHI) using respective key-1 for each i-DNA. (a) i-DNA 1, (b) i-DNA 2, (c) i-DNA 3, and (d) i-DNA 4. (e-h) qPCR measurement of Eve’s DNA before (red) and after (black) key-2 treatment using respective key-1 for each i-DNA. (e) i-DNA 1, (f) i-DNA 2, (g) i-DNA 3, and (h) i-DNA 4.
d-DNA 2-BamHI
d-DNA 2-EcoRV

CTCATCCGCTGTCCTCGAACATATACCGGATATATCTTGACCGACGACATCAACACTCCAGGGT

TCATCCGTGTCTGTACCAGCTTGATATCTCTAGTGACGACGACATCAACCTCAAGGGT

ACCGCTGAGTTGATGTCCTCAACGGGATATCTCTCGAAATTTGACTACAGACAAGCGGATGAG

ACCTGAGTTGATGTCCTCAACGGGATATCTCTCGAAATTTGACTACAGACAAGCGGATGAG

CTCATCCGTGTCTGTACCAGGACACATCAACCTCAAGGGT
d-DNA 3-BamHI
d-DNA 3-EcoRV
d-DNA 4-EcoRV
d-DNA 4-EcoRV
Figure S7. Sanger sequencing chromatogram of 50 plasmids isolated from 50 randomly picked colonies. Sequencing was performed using pJET1.2 forward sequencing primer. All chromatograms were sorted into each type of d-DNA (d-DNA 1 EcoRV, d-DNA 2 BamHI, d-DNA 2- EcoRV, d-DNA 3-BamHI, d-DNA 3-EcoRV, d-DNA 4- BamHI, and d-DNA 4-EcoRV).

2. Program of simulation for Figure 4f

```cpp
#include <Carbon/Carbon.h>
#include <iomanip>
#include <ctime>
#include <iostream>
#include <fstream>
#include <sstream>
#include <math.h>
#include <cstdlib>
#include <ctime>
#include <string>
#include <stdio.h>
#include <stdlib.h>
using namespace std;

std::string rs;

int main()
{
    srand((unsigned)time(0));
    int i;
    int check;
    int n1, n2, n3;

    n2=1000;
    for (int n3 = 10000; n3 > 10; n3 = n3-200){
        n1=0;
        for (int r1 = 0; r1 < n2; r1++){
            check = 0;
            for (int r2 = 0; r2 < n3; r2++){
                i = (rand()%4)+1;
                if (i == 1) {rs += 'A';}
                if (i == 2) {rs += 'C';}
                if (i == 3) {rs += 'G';}
                if (i == 4) {rs += 'T';}
            }

            std::size_t found = rs.find("ACGTAT");
            std::size_t found2 = rs.find("TCAGTA");
            std::size_t found3 = rs.find("GTACGGTG");

            if
```
3. Supplementary tables

**Table S1 Sequence of oligonucleotides comprising DNA steganography**

| Oligonucleotides | Sequence (5' to 3') |
|------------------|---------------------|
| i-DNA1           | GACAATTCACACGTCCGCAGTCTGACGTGACATGAATGAGAAGAGGAGCGTGG |
| d-DNA1-Smal      | GACAATTCACACGTCCGCNNNNNNNCCGCGNNNNATAGAGATCGAAGAGAGCGTGG |
| d-DNA1-EcoRV     | GACAATTCACACGTCCGCNNNNNNNGATATCNCCGNNNNAGAAGAGATCAACTCCAGG |
| i-DNA 2          | CTCATCCGCCTCTGACCTAATCTCCTGACATTAACGCGCAAGAAGAGATCGCTGAGG |
| d-DNA 2-EcoRV    | CTCATCCGCCTCTGACCTAATCCTAAGACATTAACGCGCAAGAAGAGATCGCTGAGG |
| d-DNA 2-BamHI    | CTCATCCGCCTCTGACCTAATCCTAAGACATTAACGCGCAAGAAGAGATCGCTGAGG |
| i-DNA 3          | CGGCTACCCCAAGGAGATCGAAGAATCACTCCAGG |
| d-DNA 3-EcoRV    | CGGCTACCCCAAGGAGATCGAAGAATCACCTCAGG |
| d-DNA 3-BamHI    | CGGCTACCCCAAGGAGATCGAAGAATCACCTCAGG |
| i-DNA 4          | TGTCCCGATGACGTAACGATCGAAGAAGGAGATCTGACGTAAGG |
| d-DNA 4-EcoRV    | TGTCCCGATGACGTAACGATCGAAGAAGGAGATCTGACGTAAGG |
| d-DNA 4-BamHI    | TGTCCCGATGACGTAACGATCGAAGAAGGAGATCTGACGTAAGG |

**Table S2 Primer Sequence**

| Primers        | Sequence (5' to 3') |
|----------------|---------------------|
| key-1-DNA-1-REV| CGACGCTCTCCGACGATCCAT |
| Key-1-DNA-FOR  | GACAATTCCACAGCGTCGCG |
| Wrong key-1-R  | GAGATCGAAGAGGAGCGTGG |
| Wrong key-1-F  | TGTCTCCAGCGCCCTAT |
| Key-1-DNA-2 REV| ACCGCTCCGAGGAGGAGCTG |
| key-1-DNA-2 FOR| CTCCTCGGCGGTCGAGCTC |
| Key-1-DNA-3 REV| CGGAGGACCCGAGGATCTC |
| Primer          | Sequence          |
|----------------|-------------------|
| key-1+i-DNA-3 FOR | CGGCTACCAAGTGAGAT |
| Key-1+i-DNA-4 REV   | ACCCTCGATGTCGGATGG |
| key-1+i-DNA-4 FOR    | TGTCCAGGATGGCTAAAGG |