Nucleosides, Nucleotides, and Nucleic Acids, 26:1353–1357, 2007

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770701533909

Taylor & Francis
Taylor & Francis Group

EVALUATION OF DIFFERENT CHEMICAL STRATEGIES FOR CONJUGATION OF OLIGONUCLEOTIDES TO PEPTIDES

Igor A. Kozlov, Peter C. Melnyk, John P. Hachmann, David L. Barker, Michal Lebl, and Chanfeng Zhao □ *Illumina, Inc., San Diego, California, USA*

□ *We developed novel assays for high-throughput detection of one or many kinases or proteases. The assays use hundreds of different peptide substrates, each covalently linked to an oligonucleotide tag. After incubation with sample, the pool of substrates is hybridized to a microarray containing oligonucleotides complementary to the tag sequences. We screened several specific chemistries for the conjugation based on the following criteria: easy derivatization of oligonucleotides and peptides; high efficiency of the conjugation reaction; good stability of the conjugates; and satisfactory conjugate performance in our assays. We have validated selected method during the successful generation of thousands oligonucleotide-peptide conjugates.*

Keywords Oligonucleotide-peptide conjugation; protease assay; kinase assay; proteomics

INTRODUCTION

Proteases and kinases play critical roles in fundamental cellular functions. Characterization of these enzymes has been important in understanding cell biology and in identifying new drug targets. Higher throughput analyses of these enzymes are needed for the rapid identification of new substrates, inhibitors and functions for proteases and kinases. We developed a miniaturized, highly-multiplexed assay for the study of proteases and kinases in complex mixtures. The assay is based on Illumina's BeadArray platform, which employs silica beads derivatized with specific oligonucleotide sequences and seated in etched micro-wells of fiber optic bundles.[1,2] Applying unique DNA tags to individual peptide substrates leverages the convenience and simplicity of a universal DNA microarray. It was crucial for the success of the assays to adopt a robust method for the generation

The protease project was partially funded by NIH grant R44 AI056869-02; the kinase project was partially funded by NIH grant R43 GM071272-01A1. We thank Melissa Shults, Nicholas Nelson, Joe Musmacker, Anu Srinivasan, Veronica Shevchenko, Bahram Kermani, and Gali Steinberg-Tatman for their help with this project.

Address correspondence to Chanfeng Zhao, MyChem, 11494 Sorrento Valley Rd., Suitek, San Diego, CA 92121. E-mail: Chanfeng@san.rr.com

of oligonucleotide-peptide conjugates. We chose to conjugate deprotected oligonucleotides and peptides in solution since currently there is no commercially available chemistry for the synthesis of DNA-peptide conjugates on solid supports. The conjugation reaction has to be very specific and does not involve any groups that can be presented on the side chains of peptides. Since carbonyl group addition reactions provide a good tool for the specific generation of nucleic acid conjugates,[3] 5′-aldehyde modified oligonucleotides were chosen for the conjugation and the possibility to use peptides with various N-terminal modifications was investigated. Several criteria for the oligonucleotide-peptide conjugation method were evaluated such as easy derivatization of incoming oligonucleotides and peptides, high efficiency of the conjugation reaction, good stability of the conjugates, and satisfactory conjugate performance in our assays.

RESULTS AND DISCUSSION

Protease and Kinase Assays

We have developed solution-phase assays for the detection of protease and kinase activity by measuring the signal from over one thousand unique peptide substrates simultaneously. In the protease assay a mixture of peptide-DNA conjugates is treated with proteases or a biological sample in solution. All peptides have a biotin residue at the C-terminus and are conjugated to their respective DNA tag via the N-terminus that always ends with five histidine moieties. Following the protease reaction, the sample is subjected to a streptavidin pulldown that leaves only the cleaved conjugates in solution. Cleaved conjugates with DNA tags are hybridized to Illumina's universal Sentrix Array Matrix; signal is amplified and detected using antibodies to the His5-moiety. In the kinase assay, we study the solution-phase phosphorylation reaction of a pool of DNA-peptide conjugates by a purified kinase or cell extract. For the selective labeling of the phosphorylated residues, carboxylate moieties on the peptide side chains are first blocked with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Next, the phosphate residues are labeled with an amine-functionalized dye (fluoresceinyl glycine amide is preferred). The conjugates are then hybridized to Illumina's universal Sentrix Array Matrix; signal is amplified and detected using antibodies to the fluorescein moiety. The complete description of protease and kinase assays together with their biological performance results will be published elsewhere.

DNA Preparation

DNA synthesis (23-24 mers) was performed using phosphoramidite chemistry at 0.1 μmol scale on a 96 well plate Oligator synthesizer

(Illumina).[4] To introduce a 5′-aldehyde group onto an oligonucleotide, we attached 4-formylbenzoic acid (0.1M) to a 5′-terminal amino group of protected oligonucleotides. The reaction was carried out with the oligonucleotides on CPG using an O-benzotriazole-N,N,N′ ,N′-tetramethyl-uronium-hexafluoro-phosphate (HBTU, 0.1M) catalyzed coupling reaction in acetonitrile or DMF, containing two equivalents of diisopropylethylamine. More than 90% of the amines were converted into aldehydes as observed after four consecutive modification reactions (10 minutes each). The resulting 5′-terminal aldehyde group is stable during ammonium hydroxide cleavage and deprotection.

Peptide Preparation

Peptide synthesis (6-15 amino acid residues) was performed using Fmoc-chemistry at 2 μmol scale on a 96 well plate automated peptide synthesizer (Illumina).[4] Peptides were made on Biotin resin (for the protease assay) or on Rink resin (for the kinase assay) from Novabiochem (USA). Peptides for the protease assay have a His5 tag at the N-terminus. At the end of the synthesis Boc-protected aminooxyacetic acid (Novabiochem) was attached using standard peptide coupling conditions or other aldehyde reactive groups as shown in Figure 1.

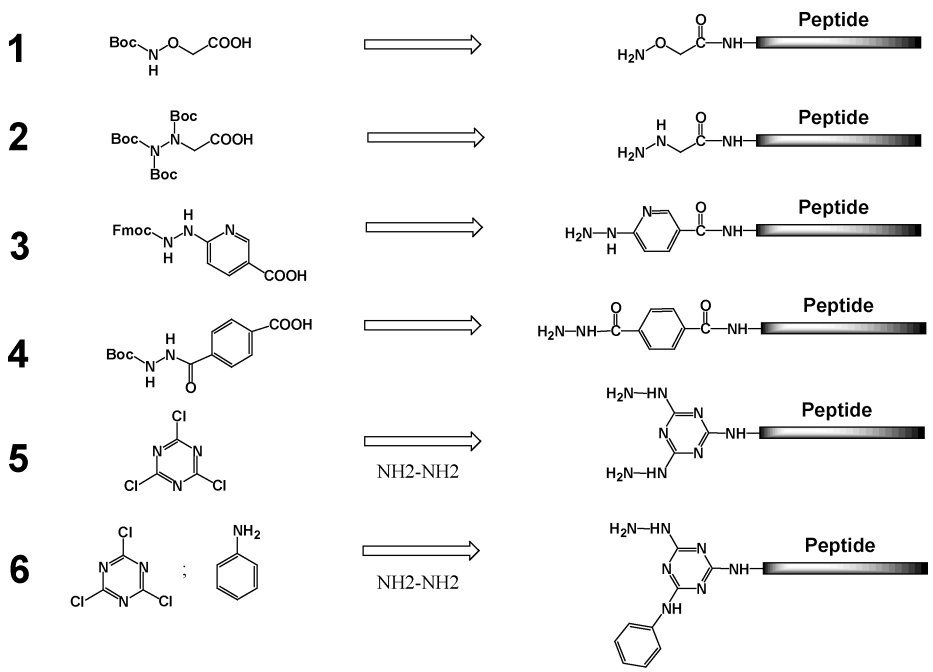

FIGURE 1 Different aldehyde reactive groups were attached to N-terminus of peptides for the conjugation to 5′-aldehyde labeled oligonucleotides.

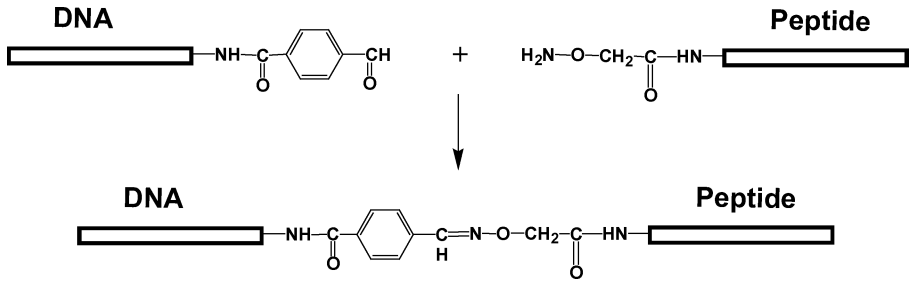

FIGURE 2 The scheme of DNA-peptide conjugation reaction that was chosen for the generation of conjugate pools (1000-plex) for the protease and kinase assays.

DNA-peptide conjugation. Oligonucleotides with a 4-formylbenzoic acid residue attached to the 5′-end were used for conjugation to peptides. In order to find the best aldehyde reactive group for peptide modification six different aldehyde reactive groups were attached to the N-terminus of a peptide (Figure 1). The conjugation reaction is efficient for all peptide modifying groups tested (over 90% yield of conjugate). To test the stability of the DNA-peptide linkages, the purified conjugates (1-6) were incubated in 50 mM Tris-HCl buffer, pH 7.8, containing 100 mM NaCl for 24 hours at 37°C and analyzed using 15% 7M urea PAGE . No significant degradation product (starting DNA) was detected in conjugates (1) and (3). Conjugates 5 and 6 produced less than 5% starting DNA, while conjugates 2 and 4 resulted in about 10% of starting DNA. A conjugation method that uses an aminooxyacetic acid residue (Figure 1, #1) attached to the N-terminus of peptides was chosen for generation of large numbers of DNA-peptide conjugates. The scheme of the reaction is shown in Figure 2. The conjugation reaction is very efficient; the materials used to modify the DNA and peptides are not expensive; and the resulting conjugates have great stability. The time course of the reaction is shown in Figure 3. The reaction proceeds well at 200 uM oligonucleotide, 600 uM peptide in 0.2M Na-citrate buffer, pH 5, 16 hours, at room temperature. The conjugates can be also purified in groups of 96 using preparative 15% 7M urea PAGE.

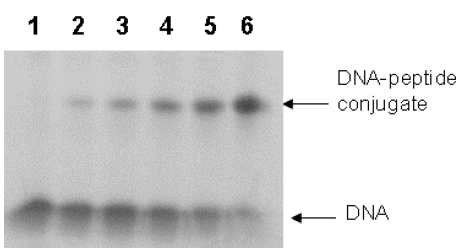

FIGURE 3 15% 7M urea PAGE analysis DNA-peptide conjugation reaction mixtures. Lane 1 – control DNA; lanes 2–6 are 1 hour, 2 hours, 4 hours, 8 hours, 16 hours reaction time.

CONCLUSIONS

Using 5′-aldehyde modified oligonucleotides and peptides with an N-terminal aminooxyacetic acid residue is the best choice due to the following reasons: 1) Large numbers of incoming oligonucleotides and peptides can be readily modified with commercial available aldehyde phosphoramidite and aminooxyacetic acid; 2) the conjugation reaction proceeds with high efficiency as demonstrated in Figure 3; 3) the obtained conjugates are stable during both the protease and kinase assay conditions; 4) the conjugates show satisfactory performance in our assays.

The conjugation reaction was validated during generation of about three thousand DNA-peptide conjugates. DNA-peptide conjugate pools (1000-plex complexity) were prepared for both the protease and kinase assay applications. The conjugate pools were used in solution-phase assay strategies to study enzymatic activity from two very distinct classes of enzymes. The strategies described leverage the universal utility of the Illumina BeadArray platform. The ability to multiplex the assay with large numbers of substrates yields unique signatures from the different enzymes, allowing complex samples to be assayed, including enzyme mixtures and biological samples. In addition, the high-level substrate-multiplexing simplifies characterization of enzymatic cross-reactivity.

REFERENCES

1. Steemers, F.J.; Ferguson, J.A.; Walt, D.R. Screening unlabeled DNA targets with randomly ordered fiber-optic gene arrays. *Nature Biotechnology* **2000**, 18, 91–94.
2. Oliphant, A.; Barker, D.L.; Stuelpnagel, J.R.; Chee, M.S. BeadArray™ technology: Enabling an accurate, cost-effective approach to high-throughput genotyping. *Biotechniques* **2002**, 32, S56–S61.
3. Zatsepin, T.S.; Stetsenko, D.A.; Gait, M.J.; Oretskaya, T.S. Use of carbonyl group addition-elimination reactions for synthesis of nucleic acid conjugates. *Bioconjugate Chemistry* **2005**, 16(3), 471–489.
4. Lebl, M. Centrifugation based automated synthesis technologies. *JALA* **2003**, 8(3), 30–35.