Development of novel chemical reagents for reliable genetic analyses

— Process from an original idea to marketing of a chemical product used for life science —

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High performance genetic analysis is an integration of various inter-correlated technologies. Of all the technologies, chemical reagents are indispensible for modifying DNA or RNA, yet the total performance of genetic analysis is sometimes limited by the insufficient capability of reagents. We have developed novel chemical reagents to increase accuracy and sensitivity in genetic analysis. We describe the development process from obtaining the original idea to marketing of the products and discuss important factors in the process.

Keywords : Gene, genetic analysis, DNA, RNA, immobilization, detection, labeling, amino group

1 Goal of the research

Genes are the common language in all organisms and viruses, and decoding them to understand their functions is necessary to learn their essence. The technologies for decoding and analyzing genes were built on the accumulation of past researches. For example, the genetic decoding of an individual has recently reached a level where it can be completed within one hour[1], and it is also possible to analyze the massive and complex inter-genetic networks as well as the gene-protein networks[2]. With the development of such highly advanced genetic analysis technology, the genetic information are utilized in wide-ranging aspects of our social lives including medicine, foods, and security, as well as basic research. In the future, this technology is expected to advance even further with the progress in the engineering fields as well as the bioscience fields. At the same time, genetic information is expected to become more closely linked to our lives, and is likely to have greater influence on our society. Therefore, both high precision and high performance are required in the genetic analysis technology. However, it is not necessarily true that all elemental technologies integrated in genetic analyses have advanced to the same level as the evolving analysis systems with higher functions. Therefore, we focused on the performance of the chemical reagents, which are essential for the genetic analyses, and conducted research to improve the overall precision of the genetic analysis by enhancing the function of the reagents.

2 Scenario to realize the goal

Most of the genetic analysis methods are organized as a system where several current technologies are integrated around an innovative core technology. Here, parts of the existing elemental technologies are often used commonly in different genetic analysis technologies. The relationship is shown schematically in Fig. 1. For example, the "synthetic DNA" that binds to the target gene and the "labeling reagent" used for highly sensitive gene detection are representative elemental technologies used commonly in many genetic analyses.

Many researches are conducted to develop some novel core technology with much funds. However, some new problems might occur in the conventional technologies when they are linked up to the core technology, resulting in the decrease of precision of the whole system. Thinking that improvement and modification of the performance of a technology with high commonality may set off a ripple effect throughout several genetic analysis systems, we turned our attention to the technologies in overlapping fields and reviewed the issues. As a result, we focused on the aforementioned technologies of "synthetic DNA" and "labeling reagent", and challenged improvement in the precision of the overall genetic analyses.

3 Synthetic DNA linker and labeling reagent

From the 1990s to present, advanced genetic analysis devices such as the DNA chip (microarray) and next-generation high-speed sequencers have been developed, and these technologies continue to advance as we speak. In most of these analysis systems, the probe DNA (oligonucleotide) that binds sequence-selectively to the target gene is immobilized onto a solid surface of flat plates or microbeads. The immobilization is achieved by the covalent bond between the special chemically modified linker incorporated into the DNA and the reactive groups of the substrate surface (Fig. 2a). The linkers

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are also used to conjugate with fluorophores or drugs to the DNA (Fig. 2b). Under such circumstances, we determined that the "linkers" played an extremely important role among the "synthetic DNAs". Similarly, we thought that the reactivity of the reagents to "label" genes recovered from samples not only would affect the sensitivity in detecting minute amounts of genes (Fig. 2c), but also may lead to the development of nucleic acid drugs. Therefore, we focused on the issues in conventional linkers and labeling reagents, and aimed to develop a new type of reagent with higher performance. After describing the development and the product realization of the linker used for probe modification, we shall present the development of the labeling reagent.

4 Development and results

4.1 Development of the amino linker

4.1.1 Goal of the development

The DNA used as a probe is synthesized sequentially by coupling the four monomer building blocks - adenine, guanine, cytosine, and thymine, according to sequence information. At the same time, the linker needed for the immobilization of the DNA to solid surface is incorporated to the termini of synthesized DNAs by using the specific "linker reagent" at the final step of the synthesis. Although there are several types of linkers possessing the different functional groups, the amino linker with primary amino group is used most frequently for the chemical modification of DNA because of the chemical stability and the easy handling.

DNAs modified with the amino group (amino-modified DNA) are generally conducted by specialized DNA synthesis companies. In one process of this synthesis, defective DNAs failing in the linker incorporation must be separated from amino-modified ones. However, since the presence of an amino group presents only a slight chemical difference, it is difficult to conduct this separation in a short time period. On the other hand, due to the recent demand for comprehensive genetic analysis of whole genomes, it has become necessary to prepare several hundreds to several thousands of amino-modified DNAs in parallel. However, we imagined that the DNA synthesis companies were facing trouble in their synthesis and purification processes due to the use of the conventional linker. Also recently, there is an increased potential for oligonucleotide drugs such as aptamers and siRNA. In order to increase their functions *in vivo*, various functional compounds must be conjugated with oligonucleotides through the linker with high yields, and we thought there would be a high demand for increasing the reactivity of the amino group as well as simplifying the purification process (Fig. 2B, Table 1).

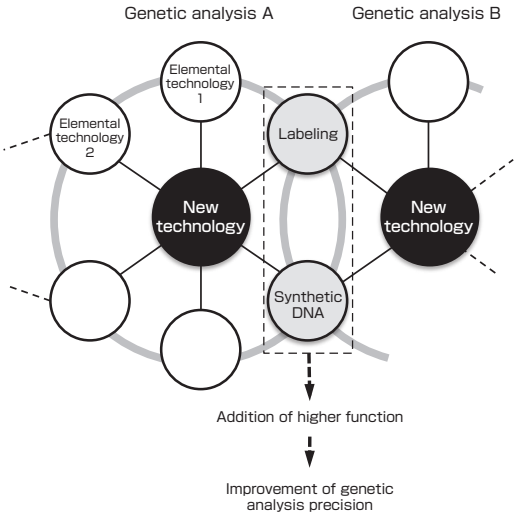

Fig. 1 Technological configuration of the genetic analysis system

The conventional technologies such as synthetic DNA (RNA) and labeling are used commonly in several genetic analysis systems, and also are related to pharmaceuticals.

Table 1 Advantages and disadvantages of amino linkers

	Conventional	First-generation	Second-generation
Advantages	• Stability • Cost • Performance	• Reactivity (> Conventional) • Purification (≧ Conventional)	• Reactivity (> Conventional) • Purification (≧ Conventional)
Disadvantages	• Purification • Reactivity	• Stability • Cost • Performance	• Cost • Performance

Therefore, we decided to develop a new amino linker that enabled both the high-purity high-throughput purification of amino-modified DNA (or RNA) as well as the high chemical modification efficiency, so it could be utilized in the recent comprehensive genetic analysis and the nucleic acid drugs.

4.1.2 Development of the first-generation amino linker

The conventional amino linker has a simple structure where a primary amino group is linked to the terminal of the straight carbon chain. We first developed a series of amino linkers which consist of a single aromatic molecule (Fig. 3, first-generation amino linker). We expected that the reaction efficiency would be enhanced by hydrophobic interaction between the aromatic residue and the target molecule. Also, we expected that the separation using the reverse-phase column chromatography would become easy because of the increased hydrophobic property of the amino-modified DNA molecule.

Several types of amino linker reagents with different distances between the aromatic and amino groups were synthesized (Fig. 3; L1, L2), and the chemical properties of the amino-modified DNA were examined. The first-generation amino linkers dramatically improved both efficiencies in the coupling to the primary amine and the purification compared to the conventional linker (Table 1)[3], and we applied for the patent jointly with a collaborating company for the first-generation amino linker in 2004.

4.1.3 Research for practical application

We planned the product realization of "high-performance DNA chip" with our joint research companies, by selecting the "ssN-linker" that showed the highest performance among the first-generation types as the terminal modification. The basic data concerning ssN-modified DNA was already completed at AIST, but in order to realize actual use by the bioscience users, it was necessary to clear three major issues: 1) synthesis of the linker reagent, 2) synthesis and purification of the amino-modified DNA, 3) fabrication of the DNA chip using amino-modified DNA and its performance evaluation (Fig. 4a). However, the joint research partner engaging in 3) could not conduct business in chemical synthesis of 1) and 2), and we became painfully aware of the difficulty of realizing a chemical reagent product for use in the biosciences field. Therefore, we set out to explain the function and advantage of the new linker to the chemical companies so that they would undertake the synthesis business of this linker. A certain custom chemical company showed interest in the ssN-linker, and we provided both the linker reagent and the DNA synthesis technologies to this company (Fig. 4b, c). While the technology transfer of mass synthesis of the reagent alone (1) was done easily, time was needed for the technology transfer of synthesis and purification of multiple DNA probes (2). This was because our protocol established in the AIST lab level did not directly fit with that used in the company operating synthesis and purification of several hundreds and thousands of DNAs with automatic machines. That is to say, the protocol had to be adjusted for automation. On the other hand, along with the synthesis work (1 and 2), we also worked on the fabrication of the DNA chip in which ssN-modified probes were immobilized onto the slide glass, and evaluated its function (Fig. 4b, c). Although the work of evaluating the functions of the linkers both upstream (1 and 2) and downstream (3) was extremely hard, for the actual use of the linker by the bioscience users, we believed it was necessary to establish the route and to indicate our product's superiority to the conventional technology. Therefore, we collaborated with the companies for this work. As a result, we demonstrated that our linker showed higher performance than the conventional reagents in all evaluations, and licensed the manufacture and sales of the ssN-linker modified DNAs to the DNA synthesis company, as well as the sales of DNA chip to the bioscience company.

4.1.4 Discovery of issues and interruption of product realization

For the commercialization of chemical reagents, the stability of the reagent itself and the DNA modified with the reagent are important check items. Since such stability test is generally time-consuming, it was started at AIST at nearly the same time as the technological transfer to the private sector. As a result, while the ssN-linker was stable in the form of a reagent, a slight amount of ssN-modified DNAs were decomposed to lack original property under severe heated alkaline conditions[4]. Since it was stable under ordinary conditions, it was not a major issue in practice. However, the usage and storage greatly depend on users, and we could not negate the possibility that this may develop into a critical problem after it was marketed as a product. Therefore, we decided to halt the licensing of the ssN-linker. We explained the situation to the people involved in both projects of DNA chip fabrication and DNA synthesis, and asked them to temporarily interrupt the projects. The instability of the ssN-linker was an unforeseen result. How much AIST should be involved in product realization including stability testing is a difficult issue. However, we must reflect on the point that we might have rushed the application (downstream) research involving the private companies immediately after discovering the new material, the ssN-linker.

4.1.5 Development of the second-generation amino linker

The interruption of the project was a major setback, and we also experienced a sense of defeat. However, we were

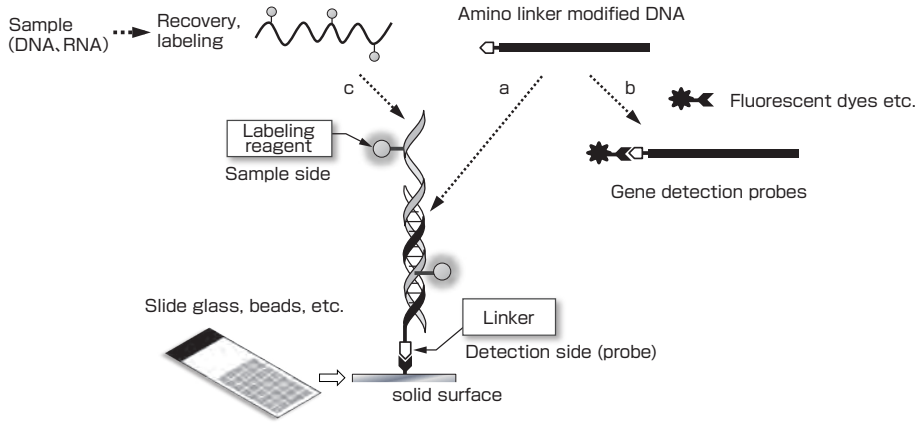

Fig. 2 Amino-modified DNA and labeling of nucleic acids

Amino-modified DNA is used for immobilization to the solid surface (a) or bonding with chemical substance (b). The sample DNA (RNA) with the complementary sequence is labeled with the labeling reagent (c).

investigating the high reactivity of the ssN-linker along with the work of product realization and the basic research turned out to be fruitful. Soon, we found that the structure of the linker alters the chemical property of the neighboring primary amine increasing the reaction efficiency with the target molecule[4]. Since this structure was a new discovery not included in the patent for the first-generation product, we applied for a new patent, and this new structure was called the "second-generation amino-linker". Although several compounds belong to the second-generation type due to the common structure, we selected the highly stable "ssH-linker" as the next linker after various investigations (Fig. 3). Unlike the ssN-linker, the ssH-linker does not contain the hydrophobic group in the molecule, but it is possible to very quickly remove the hydrophobic group that protected the amino group under moderate conditions. This chemical property of the ssH-linker enabled the high-throughput purification easily by reverse-phase chromatography, and it was also confirmed that the conjugation efficiency of this linker with the target molecule was superior to the conventional amino-linkers (Table 1, Fig. 5). Moreover, the ssH-linker was chemically more stable than the ssN-linker, and had the advantage of the synthesis cost being almost the same as the conventional reagent (Table1).

Due to such superiority, we re-proposed the ssH-linker to the DNA synthesis company and the DNA chip manufacturing company, and requested the restart of the interrupted projects. Since it became possible to keep the unit price significantly low for the ssH-linker, the chemical synthesis company accepted it smoothly, while time was needed for getting approval from the DNA chip company that had been collecting data for the DNA chip using the ssN-linker. Later, the technological superiority of the ssH-linker was acknowledged by the two companies. We concluded a licensing agreement for the domestic DNA synthesis using the ssH-linker in 2006 with the DNA synthesis company. Also, in 2007 and 2008, the DNA chips using the ssH-modified probes were commercially produced.

The ssH-linker has higher reactivity to active esters compared to conventional amino-linkers, and its modified DNAs and RNAs show high-purity by high-throughput purification. In addition, the low cost of the reagent promoted the use of the linker in the DNA synthesis companies, and it is now on market worldwide by overseas chemical companies. The license of the lower priced ssH-linker meant the reduction of royalties for us, but we decided to license it so that as many companies and research institutes use our product as possible.

4.1.6 Development after licensing

The conventional amino linker has been used throughout the world for a long time, and it has been already built into the current genetic analysis systems. Therefore, it was not easy to replace the conventional linker by the new type having a different structure. This meant that after the license, we faced the difficulty of "selling a product or getting people to buy it." To steadily increase the use of our linker, we continued application researches to propose new usages other than genetic analysis, as well as scientific demonstrations through publication of papers. As a result, we were able to propose an alternative usage of this reagent in the oligonucleotide therapeutic field[5], and the demand for linkers is recently

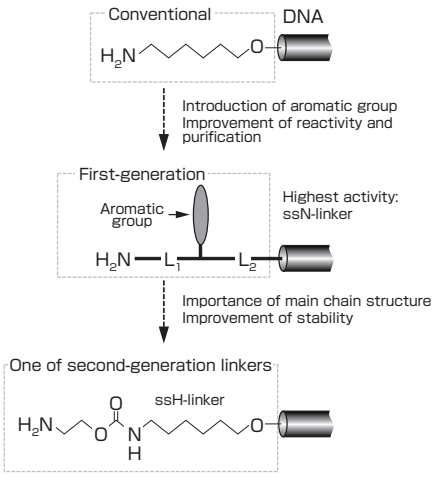

Fig. 3 Structures of the amino linkers bonded to the DNA and the flow of development

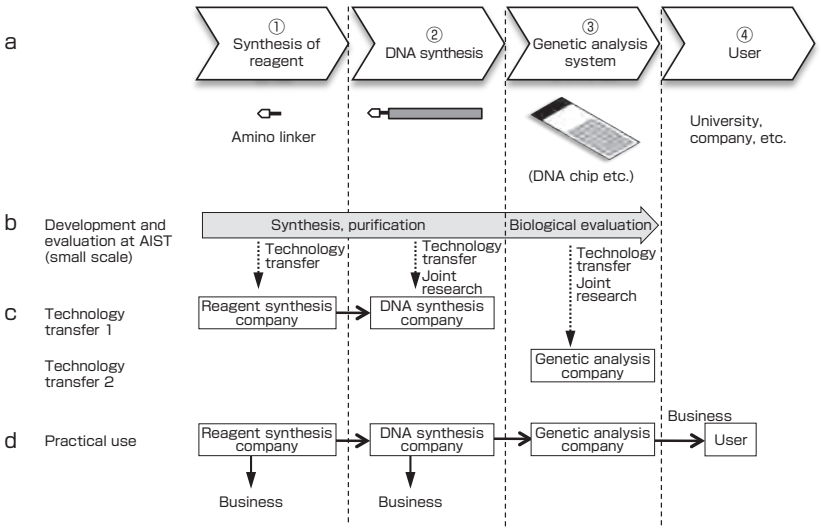

Fig. 4 Process from the linker reagent to the utilization of analysis system

Basic development and the construction and evaluation of the model system were conducted at AIST, and the efficacy of the performance was confirmed (b). Later, the technology was transferred to the private sector (c), evaluation of biological experiments were done at the genetic analysis company (c), and the route for utilizing the amino-linker reagent in bioresearch was established. Businesses are conducted for the reagent and products treated with the reagent.

rising in the new field. We feel it is important to continue R&D to seek new advantages to develop the technologies in the niche field, instead of simply ending with the licensing of the product.

4.2 Labeling reagent

In the development of the first-generation amino linker, we found that the amino group close to the aromatic residue reacts efficiently with the target molecules. To extend this principle to other issues, we planned the development of a reagent that labels nucleic acids isolated samples. Since the amino-linker is a reagent related to probe modification, we aimed to construct the complementary relationship by developing a superior labeling reagent.

To efficiently label DNA or RNA recovered from living samples, the binding efficiency of the reagents with nucleic acids is one of the important factors. While enzymes are useful for labeling of nucleic acids[6], the efficiency frequently depends on the target sequences. We thought that a labeling reagent could be a universally useful tool just like the amino linker, because it reacts with the "aldehyde groups" generated naturally or artificially in both DNA and RNA. Since there were already several marketed reagents labeling aldehydes, the developed product must have higher performance than the existing products. To obtain high reactivity to nucleic acid, we synthesized a reagent with linked aromatic group in proximity to the aminooxy group, which is known to react with the aldehyde group, using the know-how we obtained in developing the amino linker (the aromatic residue improves affinity to the nucleic acid) (Fig. 6). Since the incorporation of the hydrophobic group made the reagent poorly insoluble, we synthesized another compound which had a guanidino group of a positive charge. We expected the reagent molecule would not only increase the affinity to the negatively charged nucleic acid but also become water soluble. As a result of the reaction, the new labeling reagent with both the aromatic and the guanidino groups showed much higher reactivity to the aldehyde group in nucleic acid as compared with the commercially available reagent, and could sensitively detect aldehyde groups produced in the genomic DNA[7]. The reactivity can decrease the amounts of genes required for the analysis, and achieve highly sensitive gene detection. In addition, the synergetic effect of the aromatic and guanidino groups provided important findings for the creation of other functional molecules binding with nucleic acids. The patent was filed in 2007 for this product, and papers were presented at the academic societies in 2009. As a result, we received requests for samples from several research institutes, and its activity is under evaluation at this moment. We hope to have this labeling reagent used widely in society in the future.

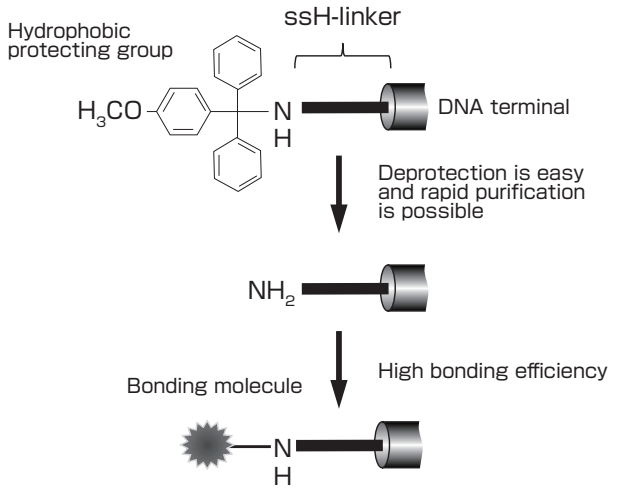

Fig. 5 Outline of the property of ssH-linker
The deprotection speed of the hydrophobic protecting group and reaction to the amino group increased.

5 Discussion

Since the "progress of the research" and "patents" were major points in our R&D, we shall discuss them in detail.

5.1 Progress of the research

The research was started in the latter half of 2003, and the reagent went on worldwide sale by 2007. There were several factors for this realization. As described above, the realization of a chemical reagent used in biological analyses must not only cover wide-ranging research fields from organic chemistry to biosciences, but solve several issues including stability and cost. To achieve valuable effects within the limited budget and human resources, we thought that a development of some universal technology was necessary. Therefore, we selected the research of the amino-linker that was used "commonly" in several analyses. While this was a

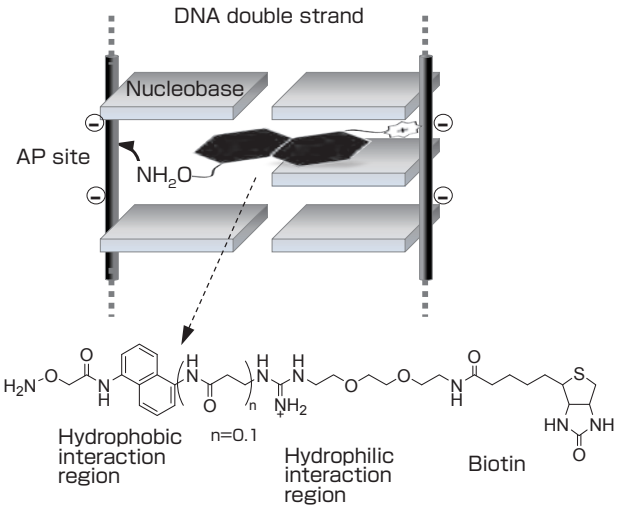

Fig. 6 Schematic diagram of the reaction with labeling reagent
The + and – indicate the positively charged guanidino group and the negatively charged phosphodiester group, respectively. The AP site is a structure of damaged DNA lacking the nucleobase, and possesses the aldehyde group.

"non-trendy topic" that had been considered to have nothing to be developed, we could perform the original researches (Factor 1). To quickly conduct the wide-ranging research, we held intense discussions with our collaborators. Such collaborations inside and outside of AIST were extremely important (Factor 2). In the improvement of the existing technology, particularly, the "speed" of the development seemed to be very important, and we carried out the research with practical use in mind from the development stage. While this may not be the best way, we believe it is very important to develop our technology speedily in life sciences which progress at an extremely fast pace (Factor 3). As the progress of our research was accelerated day by day, frictions were generated with the research environment systems. Therefore, there should be an environment that would minimize deceleration. Also, the development of the second-generation linker was important for the product realization, but this could not be accomplished without the continuous basic research on the chemical property of the first-generation linker. We realized that it was essential to pursue the basic research in practical realization (Factor 4). While four factors described above were derived as a result of the research, perhaps it was most important that we engaged in research with passion for contributing to society by producing valuable products, and joint research with private companies always reminded us that we were linked to society.

After licensing one product, we started the second research on the basis of our findings obtained from the first research (Fig. 7). However, to further broaden the potential of the licensed product, it was still necessary to continue working on the first research. Our tactics allowed quick extension of the first findings to wide-ranging fields, but two projects (the newly started research and the application of the licensed technology) had to be handled by two full-time employees, and this was extremely severe in terms of budget and physical capacity. Licensing is not the goal and researches are carried on. We should construct a research environment where researchers can carry out both basic and application researches smoothly.

5.2 On patent

For the developed reagent to be used in society, a patent is necessary in reality. In engaging in joint research with a private company, we placed extreme importance on patent from the beginning. Therefore, we were extremely careful about conducting preliminary patent survey for our project. As a result, novelty was recognized in Japan and overseas for the amino linker, the patent was accepted extremely quickly, and this led to the licensing. The patent for the labeling reagent will soon undergo patent examination, and we have received report that the developed reagent is novel.

On the other hand, much cost is required for a patent. We filed several related patents other than the main patent, but we withdrew the applications without moving on to examination requests or overseas applications for the patents that we thought would not be licensed, and narrowed down the items on our own. While patents cannot be obtained without budget, large amount of budget does not necessarily guarantee a product or a patent. Although the R&D and the handling of the patent reflect the organizational policy, we believe that if the practical use of technology is the goal, it is important to establish an environment that allows filing for

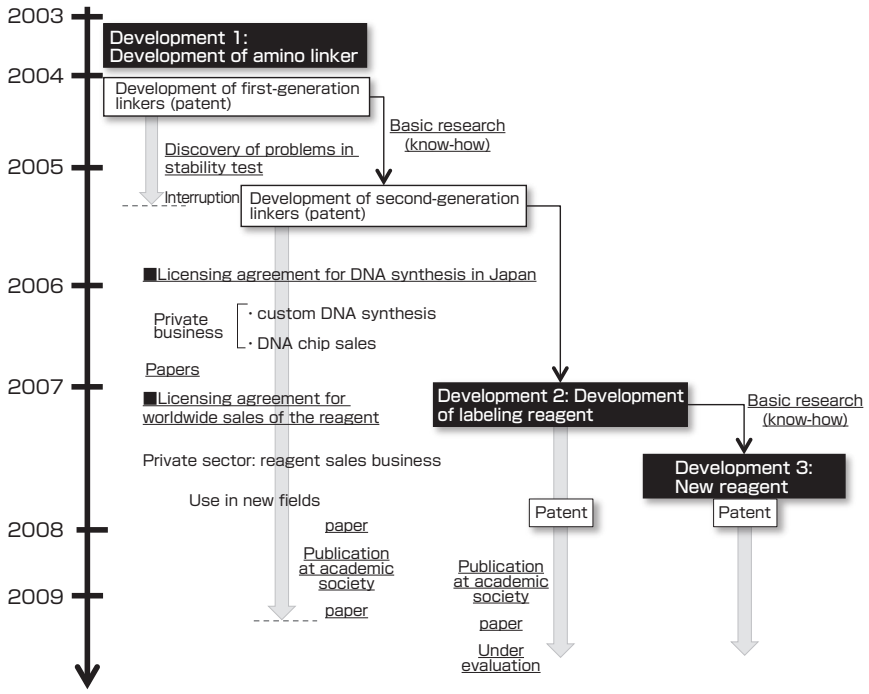

Fig. 7 Development of the R&D

good patents and supports such endeavors.

6 Issues for the future

In the development of the two types of reagents, the initial goal was achieved by conducting research to improve the performance of the existing reagents. However, we also realized that the existing technologies were deeply integrated into many systems, and time is needed for replacing them. Therefore, for the next development, we wanted to attempt an approach of proposing a totally new idea without concern for the immediate demand, and we recently developed a reagent that has unique properties (Fig. 7; Development 3). Although the details of this reagent are unpublished, we have filed for its patent. This third reagent has properties unseen before, but does not necessarily fulfill the users' specific demand. Therefore, unlike the earlier reagent developments, we have no image of the final product at this moment. For this third reagent, we must create the demand on our own, or present this technology to the world through papers and hope others will come up with ideas for its use.

Although there are numerous discussions on potential and demand (or "seeds and needs"), we have no idea which approach is the best from our experience in development. However, since unforeseen events do occur, there is the danger that if the researchers concentrate too much on potential and demand, they may become stalled and be unable to accomplish much. Both potential and demand are necessary to realize a product, and it is necessary to maintain the balance of the two in the process of research and revise them if necessary. We also believe that rather than setting licensing of the product as the goal, a venture spirit is needed for the true realization of a product.

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Discussions with Reviewers

1 "2 Scenario to realize the goal"

Comment (Yoshifumi Jigami, Research Center for Medical Glycoscience, AIST)

In this section, the explanation of Fig. 1 is difficult to understand. It may be better if you specifically explain each "elemental technology" of the genetic analysis system and their mutual relationship, as well as the relationship with "labeling" and "DNA synthesis". Also, "conventional technology", "first-generation type (ssN-linker)" and "second-generation type (ssH-linker)" of Fig. 3 should be compared in a table that shows their properties, advantages and disadvantages. You should explain

what has brought you to your "scenario to contribute to the improvement of precision of the overall genetic analysis" and explain why you "selected to fulfill the demand in the niche field" in that process. This will enable the readers to understand the authors' thinking processes.

Answer (Yasuo Komatsu)

I rewrote the text to clarify the explanation of the figure and the scenario. Also, I created a simple table that summarizes the advantages and the disadvantages of the amino linkers.

2 "*3.1.4 Discovery of the problem*"

Comment (Yoshifumi Jigami)

This is an important section that explains the motivation for the development of the second-generation amino linker and I think you should give a more detailed explanation. Particularly, there should be a more elaborate explanation of your decision and the situation why the discovered problem halted the licensing of the ssN-linker and interrupted the project.

Also, I think the significance in terms of synthesiology will be clear if you add your thinking and social situation behind "the decision to allow the developed product to be used by Japanese and overseas companies despite the decrease in royalties".

Answer (Yasuo Komatsu)

At the same time that ssN-linker showed high reactivity, it was also chemically unstable. The transformation of ssN-linker is confirmed only under limited conditions. However, the situation under which the users store the amino-modified DNA may vary greatly, and we could not eliminate the possibility that the linker may transform during storage and its function may decrease. This means that if such a compound with potential danger is released to the public sector, the company must carry the risk. Therefore, although the project was far in its course, we made a decision to develop a better linker. The background of making such a decision immediately before licensing was mainly from a thought that claims from users could be extremely troublesome to deal with, as well as a vague feeling that there was a possibility of some major problem in the future.

We considered licensing not only in Japan but overseas from the beginning of the R&D. We made this decision because having the developed technology used throughout the world will expand the possibility for evolution of this technology. For royalties, rather than AIST profiting, we thought it was more important that businesses would be conducted in the private sector as shown in Fig. 4.

3 "*3.2 Labeling reagent*"

Comment (Yoshifumi Jigami)

I think it would be more useful to the readers if you compare the property of the newly developed reagent with that of the conventional reagent and comment on how much and what kind of social impact will arise from future developments based on this new agent.

Answer (Yasuo Komatsu)

The high reactivity of the developed labeling reagent to the nucleic acid will lead to the reduction in the amount of reagent needed for the detection of minute amount of samples (reduction of background value). The characteristic in the structure of the reagent also provided an important finding in the creation of the nucleic acid recognition molecule other than the labels. I rewrote the text to emphasize these important points.

4 "*4.1 Development of the research*"

Comment (Hisao Ichijo, Tsukuba Center, Inc.)

I think it will be easier to understand and the readers will be able to see the choice of the research elements conducted in the R&D process, if you explain the process by which you arrived at the new labeling reagent.

Answer (Yasuo Komatsu)

If there was no development of the second-generation reagent, there would have been no realization, and this was a major point. As you indicated, I added the importance of this development in the discussion section.

Comment (Yoshifumi Jigami)

You state, "the fact that we selected a 'non-trendy development' turned out to be important (Factor 1)". If that is true, you should explain why this is important. You should also describe a specific situation how "the collaboration inside and outside the institute to conduct wide-ranging research for product realization (Factor 2)" has been carried out.

For the Development 3 of Fig. 7, please present a discussion on how the seeds-oriented research is different from the earlier needs-oriented research and what are your assumptions on how the difference of research methods affect the research results and what kind of ripple effect (social impact) there may be in "5 Future issues". Also, it seems you are returning to a seeds-oriented research from the earlier needs-oriented research, but as a researcher, why do you need such a return? I would like to hear the "thoughts" and "future dreams" of the authors based on this experience.

Answer (Yasuo Komatsu)

Pursuing a trendy topic means that the research may be an attempt to catch up to someone else, and may end up without ever catching up if we are lacking human resource power. On the other hand, technology that was once thought to be firmly established may develop problems due to new demands arising from changes over time. Therefore, we reviewed the technology that was considered firmly established, dug up its problems, and that led to good results for this particular case. I rewrote the part that explains the other factors that led to the success. I also added some text in Fig. 6.

For demand and potential, or "needs and seeds", we engaged in "needs-oriented" research in the first two R&D. However, as written in the text, if we are caught up by "needs" only, we must compete with (that is, replace) the similar, existing technologies. It may be difficult to replace the already entrenched technology even if the new technology is superior, and this requires time. We wanted to try the route of providing a technology with absolutely no similar product, and then move toward its product realization. Therefore, we began the development of the third reagent. However, such approach may land in a place where the demands are different. Therefore, I don't know what the best approach is, but both elements are required in practical use, and I personally think that a balance has to be struck and modifications made in the process of the research.