

Via EPOLINE

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Munich, 26 October 2020

European Patent Application No. 16784755.7-1111

based on International Application No. PCT/US2016/057073

Title: "METHODS AND KITS FOR HIGHLY MULTIPLEX SINGLE
PRIMER EXTENSION"

Applicant: Qiagen Sciences, LLC

Our Ref.: SIP16268PCTEP

Reply to the Communication pursuant to Article 94(3) EPC dated 18 June 2020

I. Claims and general matters

1. First, on a formal level, we would like to point out that we entered the EP regional phase with 15 claims, not with 6 claims as indicated on the Examination report. Since the correct 15 claims are in the EP register, we refrain from refiling and assume this was just a typing error.
2. As the Examiner has correctly indicated, the claims correspond essentially to the originally filed claims except that the number has been reduced to 15. However, we would like to point out that independent claim 1 relates to a method of performing a multiplex single primer extension reaction while independent claim 11 (previous claim 19) relates to a method of sequencing nucleic acids. The IPRP, which the Examiner refers to, is focused on claim 11 (previous claim 19) while the actual focus of the present application is on claim 1. In order to shift the focus to claim 1, we refrain from amending the claims at this time.

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3. We were pleased to note that compliance with Art. 123(2) EPC was acknowledged.

II. Comments on the Communication

1. Clarity (Art. 84 EPC)

- 1.1 In the IPRP, item VIII, claim 1 is considered to lack clarity as the Examiner considers that there is no disclosure in the description as to how a multiplex single primer extension reaction could be performed with 1000 different primers. It seems there is some confusion surrounding the combination of "single primer" and "1000 different primers." This relies on a misunderstanding with respect to the primers used in claim 1. There are 1000 different target specific primers, however each primer only binds to one target and these primers are not complimentary. The one primer bound to one target is then extended to amplify the target sequence. There is no reverse primer directed to the other end of the DNA, the amplification only takes place from one end. This difference is crucial, because it is what differentiates the claimed methods from the standard prior art methods for amplifying polynucleotides, which require a forward and a reverse primer.
- 1.2 In this regard, there is no contradiction in the dependent claims. Claim 1 specifies that the 1000 target specific primers are different. On page 9 of the application, it specifically explains that:
"In a highly multiplex single primer extension reaction, primers other than a boosting primer (if present) may anneal to different genes, different regions of the same genes, and/or different strands of the same regions of the same genes as long as none of such primers forms a pair with another primer in the same reaction that allows exponentially amplification of a portion of a target nucleic acid delineated by the primer pair. In cases where two primers in a multiplex single primer extension reaction anneal to different portions of the same strand of a target nucleic acid, preferably, the different portions are sufficiently apart from each other so that the extension of one primer does not interfere with the extension of the other primer."
- 1.3 Methods for performing the invention are described in the Examples. Please note that, as stated in the introductory portion of the patent application, both single primer extension reactions using a single target-specific primer per target region and multiplex PCR methods

using pairs of forward and reverse target-specific primers are known in the art. Specifically, as stated in the section bridging pages 1 and 2 of the description,

“Highly multiplex PCR is one of the popular methods to prepare samples for targeted sequencing. Although it is a simple yet powerful tool to enrich a large gene region, it has limited ability to detect large structural variants such as gene fusion or splicing variants because the two primers per amplicon design cannot accommodate large or unknown structural changes between the two predefined primer sites. In addition, highly multiplex PCR is difficult to be scaled up to cover very large regions such as whole transcriptome or exome in a single reactions tube. The many pairs of primers will introduce unwanted artifacts when multiplex scale increases dramatically.

A recent alternative approach to enriching target loci is single primer extensions. The single primer design allows the detection of large and unknown structural variants, and improves scalability for covering very larger panels. However, because target specificity is provided by only one primer, the overall enrichment specificity is generally much lower than PCR enrichment using two target-specific primers.

- 1.4 The aim of the present application, as will be discussed in more detail below, is to provide an improved multiplex polynucleotide amplification method that uses a single target-specific primer per target region (*i.e.*, a highly multiplex single primer extension reaction). The present invention allows for enhanced primer annealing and extension specificity, without reducing primer annealing and extension efficiency.

2. Inventive Step (Art. 56 EPC)

- 2.1 We were pleased to note that the Examiner continues to consider the claims novel over cited documents D1-D4. In addition, as will be discussed below, the claims are inventive over the cited prior art. As already mentioned above, the Examination report simply refers back to the IPRP, which focusses on previous claim 19 (new claim 11), relating to a specific application of claim 1 to sequencing. However, the application of the invention is much broader as claim 1 relates to nucleotide amplification methods in general, of which sequencing is just one subset.

- 2.2 Documents D1-D4 were cited as relevant for inventive step. Document D1, which is the inventors' own publication from 2015, relates to highly multiplex sequencing reactions by including molecular barcodes and is considered closest prior art by the Examination Division. However, D1 does not disclose amplifying polynucleotides using only one target-specific primer for each target, using MutS, or the concentration of Mg^{2+} as claimed. Thus there is simply no pointer to the subject matter of the claims in D1. D1 specifically uses a forward primer and a reverse primer for multiplex reactions, marking each strand of the amplified nucleotides individually with a barcode.
- 2.3 Documents D2-D4 all require a forward and a reverse primer to each target. None of the documents D1-D4 relates to single primer extension, and therefore none of them are actually closest prior art. The closest prior art would be a document relating to single primer extension, which is different from regular PCR requiring a target-specific primer pair, and to scaling single primer extension up to a multiplex reaction. There is no indication in any of the prior art cited that the addition of Mg^{2+} and MutS would be beneficial to single primer extension reactions, let alone to upscaling these to a multiplex reaction. Thus the technical problem to be solved over the prior art must be reformulated as providing an improved multiplex polynucleotide amplification method that uses a single target-specific primer per target region (*i.e.*, a highly multiplex single primer extension reaction).
- 2.4 As discussed above, none of D1 to D4 is directed to single primer extension that uses only a single target-specific primer per target region, let alone multiplex single primer extension. For this reason, the difficulties that arise from these methods are not discussed in D1 to D4. As mentioned in the section bridging pages 1 and 2 of the description, it is clarified that single primer extension reactions have decreased primer specificity. In order to increase specificity, the description teaches on page 2:
- One way to improve single primer specificity is to conduct the single primer annealing at more stringent conditions (e.g., at high temperature and high salt condition) and only perform polymerase extension after primer binding has reached equilibrium. However, high stringent hybridization conditions typically reduce DNA polymerase activity. In addition, performing polymerase extension after primer binding is inconvenient.*
- However, the methods used and claimed in the present application to increase specificity without reducing efficiency are completely different from the standard methods. There is no

indication of the technical problem cited above, nor of possible solutions to this problem in any one of documents D1-D4.

2.5 The above-noted technical problem is solved by the addition of at least 6mM Mg^{2+} with MutS disclosed in the present application. In paragraph 1.5 of the IPRP, it is stated that according to Example 1 and Figure 2 of the present application, the annealing and extension efficiency at 6 mM seems to be lower compared to 4 mM Mg^{2+} ; the specification at 4 mM is higher compared to 12 mM Mg^{2+} (Figure 3, the comparison is however made at different primer concentrations); and if Mg^{2+} is present at 12 mM, the use of MutS increases the specificity (Figure 4). In paragraph 1.6 of the IPRP, it is concluded that no technical effect is provided for the additional use of MutS at a Mg^{2+} concentration at 6 mM (and higher). In this regard we respectfully disagree. Figure 2 of the present application shows that increasing the concentration of Mg^{2+} improved the primer annealing and extension efficiency. Figure 3 of the present application shows that the higher Mg^{2+} concentration, however, significantly reduced the specificity of primer annealing. MutS was not included in the experiments whose results are shown in Figures 2 and 3 (see Example 1 of the present application). Figure 4 of the present application shows that adding Taq MutS in primer extension reactions together with 12 mM Mg^{2+} significantly improved primer annealing and extension specificity at no cost of primer annealing and extension efficiency as indicated by consistently good sequence uniformity. Thus it plausible that amounts of Mg^{2+} above 6 mM are beneficial. Thus the technical effect has been shown. This solution to the technical problem of the invention is by no means obvious over the prior art, especially since the technical problem to be solved over the prior art is providing an improved multiplex polynucleotide amplification method that uses a single target-specific primer per target region (*i.e.*, a highly multiplex single primer extension reaction).

2.6 We respectfully ask the Examiner to favorably reconsider the inventive step objections in light of the comments and arguments presented above.

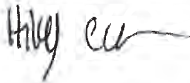
III. Requests

1. In view of the amendments and explanations set forth above, it is our view that the objections raised by the Examiner in the present Communication have been overcome and

the application can now proceed to grant. In case of disagreement, it is requested that a further Communication under Article 94(3) EPC be issued.

2. The Examiner is also kindly invited to phone the undersigned in order to clarify or discuss any concerns that the Examiner might have such that the present application could soon proceed to grant.
3. As a precaution, oral proceedings pursuant to Article 116 EPC are requested before any decision to refuse the application is taken by the Examiner.

Sincerely yours,



European Patent Attorney