

UNITED STATES PATENT AND TRADEMARK OFFICE

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**BEFORE THE PATENT TRIAL AND APPEAL BOARD**

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**ILLUMINA, INC.**  
Petitioner,

v.

**THE TRUSTEES OF COLUMBIA UNIVERSITY  
IN THE CITY OF NEW YORK**  
Patent Owner.

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IPR2018-00291 (Patent No. 9,718,852)  
IPR2018-00318 (Patent No. 9,719,139)  
IPR2018-00322 (Patent No. 9,708,358)  
IPR2018-00385 (Patent No. 9,725,480)  
IPR2018-00797 (Patent No. 9,868,985)

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**SECOND DECLARATION OF FLOYD ROMESBERG, PH.D.,  
IN SUPPORT OF PETITION FOR *INTER PARTES* REVIEW**

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| ILLUMINA Ex. 1119<br>ILLUMINA v. COLUMBIA<br>IPR2018-00291, -00318, -00322,<br>-00385, and -00797 |
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1. I, Floyd Romesberg, Ph.D., submit this declaration in response to issues and arguments raised by Columbia in this proceeding. My qualifications are set forth in my initial declaration in this matter, and I incorporate them herein.

2. In forming this opinion, I have considered the materials referenced in this declaration, as well as the materials set forth in Exhibit 1138 to this declaration.

3. I understand that Columbia has raised a number of factual issues and scientific arguments that it asserts undermine the obviousness of the claims at issue in this inter partes review. I respond to these issues below.

**The state of the sequencing art**

4. In the early- to mid-1990s, sequencing was typically focused on a version of the Sanger sequencing method. This method was also known as capillary sequencing or “first-generation” sequencing. This sequencing approach is generally outlined in Prober. Ex. 1014.

5. The preeminent sequencing effort at the time was the Human Genome Project, a multi-government-funded collaboration between a number of different entities with the goal of sequencing a human genome. The first draft of the human genome was released in 2000, and the genome was declared complete in April 2003.

6. The methods used to sequence the first human genome were very expensive, with the project estimated to have cost billions of dollars. In the late 1990s, and into the new millennium, scientists started to use the experience

generated from the Human Genome Project to develop less expensive sequencing methods.

7. Government funding agencies, among others, began to fund research and development of new sequencing methods. One source of funding at the time was the \$1000 genome grants administered by the National Human Genome Research Institute (NHGRI). The goal of these grants was to encourage research that would reduce the cost of sequencing a genome from billions of dollars to \$1000, the estimated cost at which whole genome sequencing could be used to make health care decisions. This goal, first articulated in the early 2000s, was finally met by the Illumina HiSeqX Ten product introduced in 2014.

8. In the early 2000s, different approaches were being explored as ways to devise improved sequencing techniques. One approach was to use sequencing-by-synthesis (“SBS”), a term that includes a number of different technological approaches which are unified by their reliance on the ability of DNA polymerases to synthesize DNA.

9. I understand that Columbia has argued that SBS was a disfavored approach in the sequencing field in the late-1990s and early-2000s. In support of this argument Columbia cites certain patent documents that identify challenges associated with the use of SBS. In my experience, these documents do not accurately reflect the state of the art at the time. Instead, the cited discussions are more akin to

advocacy or justification for the particular methodology disclosed in the patent. In my experience, it is not uncommon to contrast a new technology or direction of research with existing technology in order to distinguish and justify the need for further research.

10. At the time, however, SBS was clearly an area of interest for researchers. In fact, research was being conducted into different sequencing technologies, including SBS using reversible terminators. In my experience, SBS was one of the leading candidates being considered for the next generation of sequencing technologies. One of the advantages of SBS, recognized at the time, is that the removable terminator allows a very controlled sequencing process using natural enzymatic processes. As further evidence of the skilled artisan's view of SBS at the time, I note that a large number of companies and research groups pursued this technological approach, including Solexa, Amersham, Professor Steve Quake (Stanford), ASM Scientific, Manteia, and Lynx Therapeutics.

**The \$1000 genome grants**

11. I understand that Columbia has asserted that comments made in one of the grant proposals I submitted, in conjunction with Dr. Ju, for funding from the \$1000 genome project, support non-obviousness. I disagree.

12. I understand that Columbia has quoted certain portions of the grant proposal to make it appear that I stated that the Ju group pioneered the development

of the 3'-O-allyl protecting group catalytically removed with palladium. In fact, what I actually say is “The Ju group has pioneered the development of novel dNTPs that not only bear photo-detachable nucleobase-linked fluorophores (see above), but that also bear 3'-OH protecting groups. First proposed by Metzker in 1994 [10], reversible modification of the 3'-OH allows for strict control over the synthesis beyond the modified nucleotide . . . .”. Ex. 2118 at 29. The novel portion of Dr. Ju’s approach was the photo-detachable nucleobase-linked fluorophores based on linkers containing the 2-nitrobenzyl group for SBS.

13. This type of photo-detachable group is not the subject of the claims at issue in this proceeding. Unlike the claims at issue, which require a “chemically cleavable” linker, the modified dNTPs I mentioned in the grant application used a linker that is photo-cleavable.

14. While I understood, at the time, that this type of 2-nitrobenzyl group was a new development by Dr. Ju, it did not ultimately yield a very productive sequencing system. This is because, as I noted in that same discussion in my grant proposals, Dr. Ju’s modified dNTP containing a 2-nitrobenzyl group in the linker was difficult to incorporate. *Id.*

15. Ultimately, Dr. Ju was able to achieve more success when he used an allyl group in the linker (and chemical cleavage) instead of the 2-nitrobenzyl group (and photo-cleavage). Ex. 1129 (Ju 2006) at 19635.

16. It appears that Dr. Ju's switch from his novel nucleotide to the previously known allyl-derivative is what helped him achieve his sequencing goals. *Id.* at 19639 ("Second, efficient removal of both the fluorophore and the 3'-OH capping group after the fluorescence signal detection have successfully been carried out, which increases the overall efficiency of SBS."). The allyl linker used by Dr. Ju was previously disclosed in Tsien in 1990. Ex. 1013 (Tsien) at 28.

17. In my review of the Ju patent at issue in this proceeding, there is no description of the specific groups that should be used as chemically cleavable linkers. *See, e.g.*, Ex. 1002 (Ju Patent No. 9,708,358) at 11:6-10; 14:17-21 for representative disclosures. Instead, such groups were known in the prior art, for example as disclosed by Tsien.

18. I understand that Columbia also asserts that the grant is evidence that 3'-O-allyl nucleotides are inefficient substrates for DNA polymerases. This is the section of the grant application where I (and Dr. Ju) discuss and critique Dr. Ju's 2005 paper. Ex. 2118 at 29. This evaluation is relative to the expectations in the field in the mid-2000s, and the critique in the grant application is in the context of improving existing polymerase capabilities to help achieve the commercial goal of sequencing an entire genome for \$1000 (as mentioned above, a goal first articulated in the early 2000s), a much different standard than a skilled artisan would have had before the time of the filing of Columbia's application for patent, for example in the

year 2000. Further, efficiency was understood to be a relative term implicitly defined relative to natural dNTPs, and it was well understood that efficiency of blocked nucleotide analogs that equaled the efficiency of natural dNTPs was not necessary for SBS. Thus, the relative efficacy did not discourage the use of 3'-O-blocked dNTPs in sequencing. For example, roughly contemporaneous with the grant application, Illumina was on the verge of launching a commercial sequencing system using 3'-O-blocked nucleotides.

19. The fact that there was an opportunity to improve the efficiency of the incorporation process does not undermine the fact that a skilled artisan would have recognized that such 3'-blocked nucleotides could, in fact, be used as reversible terminators for sequencing.

20. In particular, with respect to achieving a \$1000 genome, increasing the efficiency of incorporation would greatly reduce costs as it would allow for the use of less of the expensive nucleotide reagents. As of the year 2000, however, before the completion of the first human genome sequence, a skilled artisan would not have been concerned with the type of price reduction necessary to achieve a \$1000 genome. Even the first Illumina SBS sequencing system did not achieve that level of efficiency, but still sold effectively in the sequencing market. As such, the fact that a certain reagent or polymerase would likely not be sufficient to achieve a \$1000

genome would not have made it any less obvious to use 3'-O-blocked nucleotides, including 3'-O-allyl derived groups, as of the year 2000.

21. In fact, the grant application specifically seeks to improve the ability to use 3'-O-allyl nucleotides in sequencing to thereby come closer to attaining the goal of the \$1000 genome. For example, a stated goal of the grant was to “[e]volve polymerases that efficiently incorporate reversibly fluorophore tagged dNTPs with 3'-O-allyl protecting groups.” Ex. 2118 at 7. Thus, even at the time of the grant application, 3'-O-allyl protected nucleotides were identified as substrates used for the sequencing process. Far from reflecting a belief that O-allyl modified nucleotides would not work for sequencing, my grant application actually demonstrates that such nucleotides were the basis for future research in the area, and underscores the fact that I wanted to be part of that future research.

**The meaning of “allyl” to a person of ordinary skill in the art**

22. I understand that Columbia has taken the position that the disclosure of “allyl ethers” in the Tsien reference refers to an undefined genus of compounds, and not to a specific chemical structure. A person of ordinary skill in the art would have understood that “allyl” has a specific meaning and refers to a specific chemical structure that, among other things, was widely used to modify alcohols (R-OH). “R” in this structure refers to any of a wide variety of compounds with the only limitation being that the hydroxyl group (–OH) is attached to a carbon.



23. As of 2000, indeed at any relevant time, a person skilled in the art would have understood an “allyl ether” to refer to a chemical structure with the general formula of  $R-O-CH_2-CH=CH_2$ , where R is some further structure and is the same as that defined for the corresponding deprotected alcohol above. A reference to “allyl ethers” does not refer to a genus of compounds modifying the oxygen—it refers to variation of the “R” of the R-O- structure being modified. Tsien, which refers to Gigg (Ex. 1046), characterizes this reference as teaching “allele ethers” because Gigg discloses more than one R-O- attached to a  $-CH_2-CH=CH_2$  (allyl) group. Ex. 1013 at 24.

24. In each instance, however, a person of ordinary skill in the art would understand that the alcohol-containing structure being modified (R-OH) was modified with the same allyl group connected to the hydroxyl (-OH) group, namely the chemical moiety  $-CH_2-CH=CH_2$ , resulting in the corresponding allyl ether (R-O- $CH_2-CH=CH_2$ ). A skilled artisan would have understood that an “allyl ether” includes that same specific structure modifying the oxygen, regardless of what exactly R represented. Thus, in the case of a modified nucleotide, an allyl ether at the 3' position would have a structure of  $R-O-CH_2-CH=CH_2$ , where the oxygen (O) is attached to the 3' carbon of the deoxyribose nucleotide of the DNA molecule, and R is the remainder of the nucleotide.

25. A number of different references reflect this understanding. One such reference is the definitions set forth by the International Union of Pure and Applied Chemistry (IUPAC), an organization which, among other things, defines standardized nomenclature for chemical compounds. For example, IUPAC published the Nomenclature of Organic Chemistry. Ex 1099 (IUPAC). This compendium provides guidance for internationally agreed-upon nomenclature for organic compounds. *Id.* at xv. As explained in the publication, the IUPAC rules “are intended to be suitable for textbooks, journals and patents, for lexicons and similar compilations, and for indexes.” *Id.* at xvii. The IUPAC publication thus defines the words artisans use to refer to particular chemical structures.

26. In my experience, the IUPAC nomenclature represents the standard terminology used to describe chemical compounds. With respect to unsaturated hydrocarbons (e.g., organic molecules containing a double bond), the IUPAC nomenclature uses the name of the hydrocarbon in conjunction with “the endings ‘-enyl’, ‘-ynyl’, ‘-dienyl’, etc.” Ex. 1099 (IUPAC) at 13. IUPAC, however, retained three exceptions to this general naming convention: vinyl (for ethenyl), allyl (for 2-propenyl), and isopropenyl (for 1-methylvinyl). *Id.* Thus, IUPAC explicitly recognizes that the term allyl refers to a specific chemical structure: 2-propenyl. In fact, this understanding was so widely held by skilled artisans in the field that IUPAC retained the term allyl to refer to the structure  $\text{-CH}_2\text{-CH=CH}_2$  instead of assigning it

a new name according to the articulated general standardized naming convention. Ex 1099 at 13; see also *id.* at 305 (explaining allyl is “preferred to 2-propenyl” and corresponds to the structure “CH<sub>2</sub>:CH·CH<sub>2</sub>—”). A person of skill in the art would have known that an allyloxy means R-O-CH<sub>2</sub>-CH=CH<sub>2</sub>; it is simply an “allyl” attached to an “oxy” (oxygen).

27. In my experience, IUPAC’s definition of allyl as a specific functional group is consistent with the way the term is used by a skilled artisan. In fact, “allyl” is used consistently over a wide range of references, including dictionaries and treatises, to refer to the same single chemical structure. Ex. 1101 (Greene and Wuts) at 67; Ex. 1102 (CRC Handbook) at 2-71; Ex. 1103 (McGraw-Hill Dictionary of Chemistry) at 24; Ex. 1039 (Vollhardt) at 503; Ex. 1104 (Solomons) at 254; Ex. 1105 (Morrison) at 209. As a result, in my opinion, a skilled artisan would understand the disclosure of an allyl ether to refer to a specific modification of a hydroxyl group with the chemical structure -CH<sub>2</sub>-CH=CH<sub>2</sub>.

**Palladium catalyzed removal of an allyl group commonly proceeds with high efficiency and yield in water**

28. I understand that Columbia has asserted that, as of the year 2000, a skilled artisan would not have understood that an allyl group could be removed using mild conditions with a high yield. I further understand that the basis for Columbia’s assertion is that, in Columbia’s view, a skilled artisan would believe that palladium

catalyzed removal of an allyl group from an alcohol would not proceed in an aqueous (water-containing) system. I disagree.

29. Well before the year 2000, a person of ordinary skill in the art would have been aware of a number of different ways to remove an allyl group to reveal an alcohol (R-OH). In particular, a skilled artisan would have been aware of the extensive literature related to the use of palladium in its zero oxidation state ( $\text{Pd}^0$ ), including the use of water-soluble  $\text{Pd}^0$  catalysts, to remove allyl-groups from alcohols.

30. Columbia argues that a skilled artisan would have believed that adding water to the Qian deprotection reaction would result in a different palladium-catalyzed process generally known as the Wacker oxidation reaction. This is incorrect.

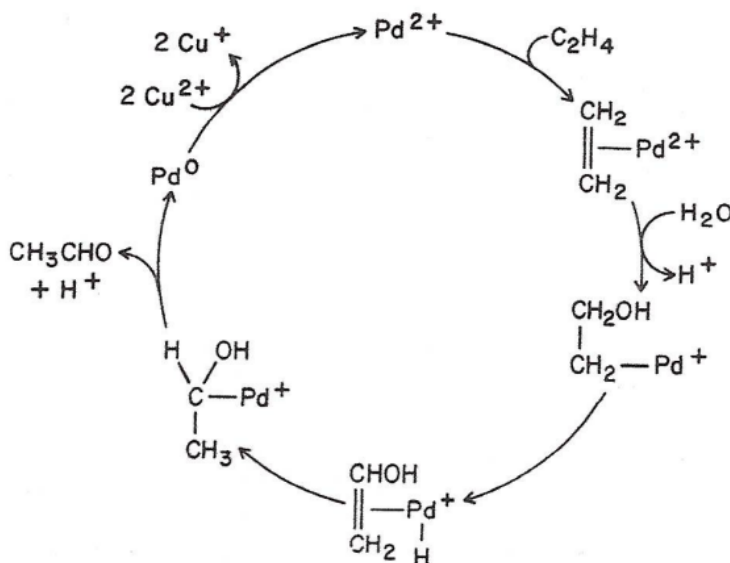
31. Palladium exists in two useful forms, an oxidized form,  $\text{Pd}^{2+}$ , and the reduced form that is relevant for the case at hand,  $\text{Pd}^0$ . Importantly, the reactivity of the two forms of palladium are very different.  $\text{Pd}^{2+}$  acts as an *electrophile* (because it has fewer electrons) and  $\text{Pd}^0$  acts as a *nucleophile* (because it has more electrons). In catalytic allyl deprotection, the palladium acts as a *nucleophile* (to form a “ $\pi$ -allyl complex” where the palladium is complexed to the  $\pi$  orbitals of the allyl group, having displaced what was previously attached to the allyl group), so  $\text{Pd}^0$  is needed. Nonetheless, these reactions are commonly performed using  $\text{PdCl}_2$  (which is  $\text{Pd}^{2+}$ )

because it is more stable as a reagent. The use of  $\text{PdCl}_2$  for  $\text{Pd}^0$ -mediated reactions was and remains a common practice, and it is possible because under most conditions some  $\text{Pd}^0$  is present (for example many solvents are thought to reduce  $\text{Pd}^{2+}$  to  $\text{Pd}^0$ ) and very little  $\text{Pd}^0$  is needed. Very little  $\text{Pd}^0$  is needed because during allyl deprotection, it acts in a catalytic fashion and is quite an effective catalyst. In some cases, to be certain that enough  $\text{Pd}^0$  is present, it is not uncommon for researchers to add reductants such as a phosphine or an amine (both can reduce  $\text{Pd}^{2+}$  to  $\text{Pd}^0$ , but both can have other functions, such as facilitating the water solubility of palladium or trapping the allyl group after it is cleaved from the alcohol). To be sure, these extra reducing agents are not always needed or used; in fact, as discussed below, the Qian reference provides an example where a sufficient amount of  $\text{Pd}^0$  appears to have been present without the addition of a phosphine or amine.

32. The Wacker process is a palladium-mediated oxidation of a double bond to yield a carbonyl compound, and it requires the palladium to act as an *electrophile* and as an oxidant. For both of these reasons, the palladium must be present as  $\text{Pd}^{2+}$ . However, because during the reaction the double bond is oxidized and the palladium is reduced,  $\text{Pd}^{2+}$  is converted to  $\text{Pd}^0$ . So the initial palladium ( $\text{Pd}^{2+}$ ) reagent is consumed and it must therefore be regenerated via oxidation of  $\text{Pd}^0$  back to  $\text{Pd}^{2+}$ . Thus, Wacker reactions are carried out with  $\text{CuCl}_2$  or  $\text{CuCl}$  and  $\text{O}_2$  added (the  $\text{O}_2$  keeps the copper in the appropriate oxidation state to re-oxidize the

palladium). In Kang (Ex. 2098), one of the references Columbia relies upon, the Wacker process is carried out using  $\text{PdCl}_2$ ,  $\text{CuCl}_2$ ,  $\text{O}_2$ , and a combination of DMF and  $\text{H}_2\text{O}$ . Ex 2098 at 4678. As explained in Kang: “The terminal allylic diol 1 was treated with a catalytic amount of  $\text{PdCl}_2$  and  $\text{CuCl}$  under an atmospheric pressure of  $\text{O}_2$ , in DMF and  $\text{H}_2\text{O}$ , at room temperature for 12 h to afford the expected methyl ketone.” Ex 2098 at 4678.

33. This Wacker oxidation reaction system ( $\text{PdCl}_2$ ,  $\text{CuCl}_2/\text{CuCl}$ ,  $\text{H}_2\text{O}$ , and  $\text{O}_2$ ) is also consistent with the other reference cited by Columbia, Parshall (Ex. 2115). Parshall, Figure 6.1, illustrates the role of copper in the oxidative Wacker process (not shown in this figure is the role of oxygen in regenerating the copper to the appropriate oxidation state to re-oxidize the palladium).



34. The Wacker oxidation reaction thus involves more than just  $\text{PdCl}_2$  and water. For the Wacker reaction to proceed, the palladium is in the oxidized  $\text{Pd}^{2+}$

form, which is generated using the presence of copper and oxygen in the system when used sub-stoichiometrically. Qian does not include copper and oxygen in its allyl deprotection reaction. Columbia does not explain why a skilled artisan would have expected Qian's reaction to proceed through a Wacker oxidation reaction in the absence of both copper and oxygen from the reaction system. In actuality, it did not. Qian taught quantitative deprotection of the allyl from the sugar hydroxyl group. Ex. 1036 (Qian) at 2185.

35. In my opinion, a skilled artisan would not have expected the use of an aqueous solvent system, without the addition of copper and oxygen, to lead to a Wacker oxidation product. As a result, in my opinion, a skilled artisan would have believed that Qian's allyl-removal could be carried out in the presence of water, without generating a Wacker oxidation product.

36. This belief would have been further supported by the fact that even though the Wacker oxidation is typically carried out in water, the same reaction pathway can take place in other solvents, including methanol. As Parshall explains, the Wacker oxidation can be carried out in other solvents including methanol, which yields "either methyl vinyl ether or  $\text{CH}_3\text{CH}(\text{OCH}_3)_2$ ." Ex. 2115 (Parshall) at 103. If the Wacker oxidation were favored relative to the allyl deprotection pathway, a skilled artisan would have expected to also observe Wacker oxidation products in methanol as well. This is not, however, what Qian reports. As Qian explains, the

result of the palladium-mediated reaction is the quantitative removal of an allyl group from an alcohol. Thus, no Wacker oxidation product was produced.

37. A skilled artisan would likewise have considered the sub-stoichiometric amount of  $\text{PdCl}_2$  used by Qian as another reason why Wacker oxidation was unlikely to occur, even with the addition of water. As disclosed by Qian, 0.5 equivalents of  $\text{PdCl}_2$  are used to quantitatively remove the allyl from a sugar hydroxyl group. Quantitative removal of the allyl group was possible because, as discussed above, the  $\text{Pd}^0$  is not consumed during the allyl deprotection reaction. Thus the palladium is catalytic, and a sub-stoichiometric amount is sufficient. In contrast, as explained by Parshall,  $\text{Pd}^{2+}$  is converted to  $\text{Pd}^0$  during a Wacker reaction, and as also discussed above,  $\text{Pd}^0$  does not engage in the Wacker oxidation reaction. Ex. 2115 at 102. Thus, as explained by Parshall, copper and oxygen are necessary in order for palladium to be used in a catalytic sense (e.g., in sub-stoichiometric amounts) in the Wacker oxidation. Ex. 2115 (Parshall) at 102. Thus, a skilled artisan would further understand that the conditions disclosed in Qian would be unlikely to yield Wacker oxidation products in view of the absence of copper and oxygen and the sub-stoichiometric amount of palladium used to quantitatively remove the allyl group.

38. Thus, in my opinion, Columbia is incorrect in stating that a skilled artisan would have believed the addition of water to the Qian allyl-removal reaction would result in a Wacker oxidation. Instead, a skilled artisan would have recognized



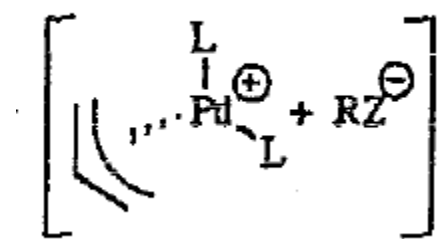
that the palladium-mediated allyl removal disclosed in Qian would likely have proceeded even in the presence of water. And obtaining a Wacker oxidation product would have required the affirmative addition of copper and oxygen to Qian's reaction, which the skilled artisan would have avoided when seeking to deprotect the allyl group and not perform Wacker oxidation on the allyl group.

39. I also understand that Columbia asserts that a skilled artisan would have no reason to believe that a water-soluble palladium reagent could remove an allyl group, for example as shown in Qian, in quantitative yield. I disagree.

40. Water-soluble palladium reagents were well known before the year 2000. For example, Lemaire-Audoire, et al. disclose that a water-soluble palladium catalyst can be prepared in situ from a combination of  $\text{Pd}^{2+}$  and TPPTS (triphenylphosphinotrisulfonate). Ex. 1114 (Lemaire-Audoire) at 8783; Ex. 1115 (Lemaire-Audoire 2) at 248. These in situ-formed palladium catalysts use “ $\pi$ -allyl methodology” (e.g., palladium as  $\text{Pd}^0$  acting as a nucleophile to produce the  $\pi$ -allyl complex, as discussed above) in the same manner as Qian to rapidly remove allyl-groups from oxygen substituents at quantitative yield in aqueous solvents (Qian does not report or study the mechanism, but I believe that a POSA would have assumed it to be an example of the  $\pi$ -allyl methodology). Ex 1114 (Lemaire-Audoire) at 8784-86; Ex 1115 (Lemaire-Audoire 2) at 249-50. As discussed above, the  $\pi$ -allyl methodology requires only a small amount of catalytic palladium to proceed, as

reflected by the sub-stoichiometric levels of palladium used in Qian to remove the allyl group. In fact, a skilled artisan would have readily understood that the reaction in Qian could be transferred to a mild aqueous environment by using the phosphine TPPTS (and possibly adding an amine to trap the allyl group and/or act as an additional reducing agent to ensure sufficient amounts of Pd<sup>0</sup> were present).

41. The palladium  $\pi$ -allyl approach is applicable to a variety of allyl derivatives, including allylic acetates, allyl ethers, allylic alcohols, and allyl amines. Ex 1135 (Yamamoto); Ex. 1134 (Takahashi). The  $\pi$ -allyl methodology was also well known for the removal of an allyloxycarbonyl groups, for example in the presence of diethylamine as an allyl scavenger, in aqueous media. Ex 1115 (Lemaire-Audoire 2) at 248. Regardless of the particular allyl derivative, the  $\pi$ -allyl methodology proceeds through the intermediate shown below (the intermediate is formed after the nucleophilic Pd<sup>0</sup> displaces the RZ group from the allyl group).



Ex 1115 (Lemaire-Audoire 2) at 248

42. In regards to the palladium-catalyzed removal of an allyl specifically from an alcohol, as is the case here, a skilled artisan would also have understood that

the use of an aqueous solvent might actually have benefits for the removal of an allyl group using the palladium  $\pi$ -allyl methodology, as compared to other solvents. This is because water is one of the most polar and hydrogen-bonding solvents known. A skilled artisan would have understood that both polarity and hydrogen-bonding stabilize an ionized intermediate, in this case the  $\text{RO}^-$  ion formed during allyl deprotection of an alcohol. By better solvating the intermediate ions, water would likely have made the reaction more favorable.

43. A skilled artisan would have recognized the general applicability of the palladium-mediated  $\pi$ -allyl approach to allyl removal in aqueous solvents, and would have expected to be able to achieve the fast, quantitative removal of allyl groups disclosed in the prior art. Ex 1114 (Lemaire-Audoire) at 8784-86; Ex 1115 (Lemaire-Audoire 2) at 249-50.

44. Finally, I note that Columbia asserts that the palladium-mediated allyl deprotection taught in the Boss reference (Ex. 1035) cannot be used to remove an allyl group because it discloses the use of perchloric acid. But Boss teaches that only “trace” (a small amount) of the relatively milder *p*-toluenesulfonic acid can be used instead of the relatively stronger perchloric acid. Ex. 1035 (Boss) at 558.

**Tsien's disclosure of an allyl capping group was not a mistaken reference to a vinyl capping group**

45. I understand that Columbia argues that Tsien mistakenly disclosed attaching an allyl group, instead of what Columbia alleges was supposed to be a vinyl group. I disagree.

46. As an initial point, I note that a skilled artisan would clearly understand the difference between a vinyl group and an allyl group. In particular, the difference is the location of the double bond relative to the substituent to which it is bound. That is, a vinyl group is  $\text{CH}_3\text{-CH=CH-}$  and an allyl group is  $\text{CH}_2\text{=CH-CH}_2\text{-}$ . A skilled artisan reading Tsien would likewise recognize that Tsien's disclosure of an allyl ether refers to a different molecule from a vinyl ether.

47. I do not agree with Columbia that a skilled artisan would look back on the Gigg reference cited by Tsien and conclude that Tsien intended to disclose a vinyl group. Gigg specifically indicates that it is directed to "The Allyl Ether as a Protecting Group in Carbohydrate Chemistry." Ex. 1046 (Title). Gigg's allyl group deprotection method is a two-step process involving isomerization followed by hydrolysis: an "allyl group is conveniently removed by [1] isomerization to the cis-prop-1-enyl group and [2] subsequent acid hydrolysis." Ex. 1046 at 1903. The molecule resulting from the isomerization to a cis-prop-1-enyl group on the sugar oxygen can be referred to as a vinyl ether.

48. Gigg recognized, however, that the acidic conditions used for hydrolysis might not always be suitable. As an alternate to acid hydrolysis as the second step in the allyl deprotection process, Gigg teaches the use of mercuric chloride in an acetone-water solution to remove the isomerized allyl group. Ex. 1046 at 1907. For example, Gigg teaches “removal of the allyl group by isomerization and mercuric chloride hydrolysis.” Ex. 1046 at 1905; see also 1906 (Compound XXXVI “was isomerized to the prop-1-enyl ether (XXXVII) . . . . Hydrolysis of the prop-1-enyl group in compound (XXXVII) by mercuric chloride gave compound (XXXVIII).”).

49. Thus, Gigg discloses the use of mercuric chloride as part of an overall scheme for removing an allyl group from a carbohydrate hydroxyl. I note that the Gigg paper was peer reviewed, and this review process would certainly have caught such an obvious error. Moreover, skilled artisans had the same understanding that Gigg’s procedure was a deprotection scheme for allyl-alcohols. For example, Boss (Ex. 1035) explains that allyl ethers are often used as removable protecting groups for sugars, and that “[r]emoval of the protecting group is usually effected by isomerization of the allyl ether to the corresponding enol ether (2) with subsequent  $H^+$  or  $Hg^{2+}$  catalyzed hydrolysis or oxidative cleavage.” Ex. 1035 (Boss) at 558.

50. As such, it is clear that a skilled artisan reading Tsien would have understood the reference to an allyl-modified oxygen to mean exactly what it says, and not as a mistaken reference to a vinyl moiety.

**Knowledge of the size of the polymerase active site**

51. I understand that Columbia has asserted that Dr. Ju was the first to understand that the enzymatic space surrounding the 3' hydroxyl is small and restrictive. I disagree.

52. Before the year 2000, skilled artisans routinely considered the three-dimensional structure of enzymes in order to better understand the type of substrates that might be used. For example, when I carried out work designing non-natural nucleotides in the late 1990s, I examined three-dimensional structures of DNA polymerases, and from those structures I understood that the space in the active site was limited. This was understood to be at least partially responsible for the specificity of polymerases that use deoxyribose substrates over ribose substrates. The difference between these two substrates (dNTPs and NTPs) is a single oxygen atom at the 2' position. Based on this ability to discriminate between very similar substrates, a skilled artisan would have understood that there was limited space at the polymerase active site.

53. This understanding is confirmed by a variety of publications that directly addressed the possibility of using 3' hydroxyl modifications. For example,

Dower (Ex. 1015) recognized that the 3' hydroxyl must be protected with small chemical structures. Ex. 1015 (Dower) at 25:48-51. Welch and Burgess (Ex. 1023) evaluated the crystal structure of the T7 polymerase and determined that large chemical structures at the 3' position cannot fit within the active site of the polymerase. Ex. 1023 (Welch) at Abstract. Likewise, Stemple (Ex. 1019) explained that the space available at the 3' hydroxyl may limit the groups that could be attached to the 3' hydroxyl. Ex. 1019 (Stemple) at 22:64-67.

54. I further understand that Columbia argues that a skilled artisan would not have considered the Pelletier reference, disclosing the three-dimensional structure of a DNA polymerase, because it was published by a group researching HIV inhibitors. I disagree. DNA polymerases are at the heart of SBS, and, in my opinion, a skilled artisan in the year 2000 would have wanted to take advantage of this three-dimensional crystal structure regardless of who published it. Three-dimensional structures determined using x-ray crystallography have been reported since the 1950s. A three-dimensional crystal structure provides a clear picture of the arrangement of non-hydrogen atoms in a protein. Sometimes three-dimensional protein structures are obtained with a substrate complexed, which helps to further define the active site where substrate binding and enzymatic catalysis takes place. Such three-dimensional structures help a skilled artisan to understand how the

substrate is accommodated within the active site, as well as what modification to the enzyme or to the substrate might make that accommodation better or worse.

55. Since three-dimensional structures are used to understand how proteins interact with substrates, three-dimensional crystal structures have been used prior to the year 2000 for the rational design of inhibitor molecules and non-natural substrates. In the case of polymerase-based anti-viral compounds, a skilled artisan would understand that many design principles are identical to the design principles for polymerase-based sequencing terminators. In particular, , polymerase-based anti-viral compounds work by being incorporated by a polymerase into a growing strand of viral DNA (or RNA) and terminating further extension. This activity is essentially identical to what sequencing terminators (both Sanger and SBS terminators) are used for – incorporation and termination.

56. To use HIV inhibitors as an example, 3'-azido-2',3'-dideoxythymidine (AZT), 2',3'-dideoxyinosine (ddI), and 2',3'-dideoxycytidine (ddC) are each converted in vivo to modified dNTPs (either the 3'-azido or the 2', 3'-dideoxy, and ddI is also converted to ddATP) that are successfully incorporated by the viral polymerase into DNA and then terminate further extension of the DNA because all three of these compounds lack a 3'-OH moiety. Ex. 1021 (Pelletier) at 1891. Notably, the in vivo activated forms of both ddC and ddA (ddCTP and ddATP, respectively) are the exact same molecules that are used as polymerase terminators



in Sanger sequencing. Pelletier's three-dimensional crystal structure in fact is a detailed atomic structure of a DNA polymerase with ddCTP bound in the active site. Ex. 1021 (Pelletier) at 1894, 1896. That is, Pelletier reports a three-dimensional crystal structure of DNA polymerase bound to a Sanger sequencing substrate, ddCTP. A person of ordinary skill in the art would have expected that this type of structural analysis, which demonstrated a polymerase's interaction with an irreversible terminator, would also have been useful for understanding a polymerase's interaction with reversible terminators. This type of structural analysis of the features of a nucleotide-derivative with respect to a crystal structure is directly applicable to the design of substrates for DNA sequencing.

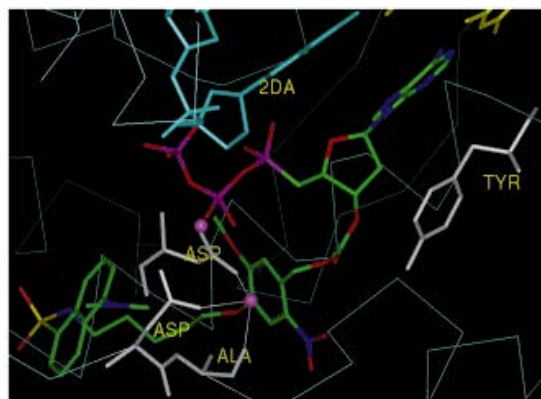
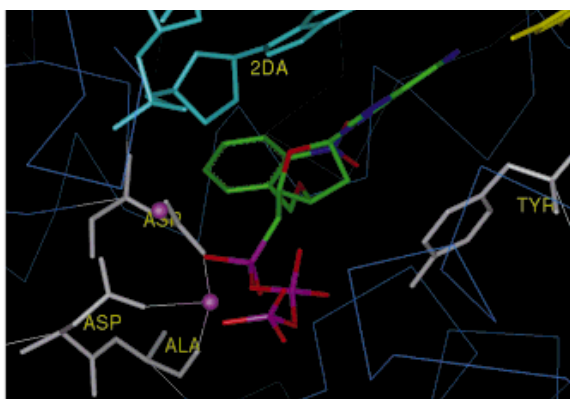
57. The Pelletier article is particularly notable, as it was published in the leading scientific journal, Science. As one of the first polymerase structures with a substrate bound in the active site, it received significant levels of attention and would have been of special interest to skilled artisans in the field of DNA sequencing, not just in the field of anti-viral drugs. Moreover, Pelletier reported crystal structures that included a DNA template-primer and ddCTP, which gives information specifically on the residues that interact with the substrates during the process of polymerase-catalyzed incorporation of ddCTP into DNA. *Id.* at 1891. A skilled artisan in the field of DNA sequencing would have understood that the ability to

examine the polymerase active site provides important information related to non-natural substrate design for improved DNA sequencing methods.

58. In fact, Tsien itself directs those in the art to look to the field of antiviral drug development for guidance on designing SBS substrates. Ex. 1013 (Tsien). Tsien explains: “Incorporation and chain termination have been demonstrated with dNTPs containing many of these blocking groups.” Ex. 1013 (Tsien) at 21. In making this statement, Tsien cites to an article, Kraevskii, that is directed to the incorporation of 3’-blocked nucleotides in the context of a viral DNA polymerase. Ex. 1013 (Tsien) at 21, 4. For example, Kraevskii teaches that “[a] comparative study of mammalian and mammalian virus DNA polymerases would be of particular importance. The differences in the properties of these enzymes provide an opportunity to block selectivity [*sic*] the reproduction of the viral genome in mammalian and human cells.” Ex. 1126 (Kraevskii) at 25. Tsien’s teachings, therefore, direct those in the SBS field to look to anti-viral drug development studies of DNA polymerase structures for the purpose of developing SBS substrates. Pelletier not only is such a study of a DNA polymerase structure, but it is a DNA polymerase structure containing a sequencing substrate in its active site.

59. Welch and Burgess also demonstrate that three-dimensional polymerase structures were considered by those in the SBS art designing substrates for SBS-based DNA sequencing. Ex. 1023 (Welch). Welch and Burgess carried out

molecular simulations using the T7 polymerase crystal structure and different dNTP analogues and discovered that many of the analogues were “too large to fit into the enzyme active site,” while a different analogue was “much less demanding.” Ex. 1023 at 951. This type of modeling is shown, for example, in Figure 3(b) and (c), which show different substrates modeled into the active site of the T7 polymerase. Ex.1023 (Welch) at 955 (shown in relative part below). Based on this modeling, using the T7 polymerase crystal structure, Welch and Burgess drew conclusions about the ability of different analogues to interact with the active site, and thus be used in a sequencing process. Ex. 1023 (Welch) at 956. For example, Welch and Burgess explained that photolabile, fluorescent protecting groups used to make 3'-O-blocked dNTPs “tend to be too big to fit into the active site of DNA polymerases,” as evidenced by both experimental results and “molecular-simulation experiments.” Ex. 1023 (Welch) at 956. This is fully consistent with the three-dimensional structural data provided by Pelletier.



Ex.1023 Fig. 3(b) and (c)

60. As the disclosures of Tsien, Pelletier and Welch confirm, those working in the field of DNA sequencing, including those working in the field of SBS, would have looked to publications of three-dimensional structures of DNA polymerases regardless of whether or not the stated purpose of such publications was the development of anti-viral compounds because the three-dimensional structures themselves provide the same information regardless of whether or not the commercial application of the information is anti-viral therapy or DNA sequencing.

**Metzker taught a skilled artisan that 3'-O-allyl modified dNTPs were incorporated by a known polymerase**

61. I understand that Columbia has taken the position that Metzker's 1994 article (Ex. 1016, "Metzker 1994") would have discouraged a skilled artisan from using a 3'-O-allyl modified dNTP as a reversible terminator in a sequencing reaction. I disagree.

62. Metzker 1994 teaches the "Termination of DNA synthesis by novel 3'-modified-deoxyribonucleoside 5'-triphosphates." Ex. 1016 (Title). In particular, Metzker 1994 teaches that eight different modified dNTPs were synthesized and tested for incorporation activity using a variety of different polymerases. *Id.* at 4259. The goal was to identify modified nucleotides that could be used "in the development of a novel sequencing strategy." *Id.*

63. Table 2 summarizes the results of the Metzker 1994 experiments. This table reports three possible outcomes for each particular polymerase-substrate pair.

First, the substrate could result in “termination,” which Metzker explains refers to incorporation of the modified nucleotide: “Termination was apparent when the reaction containing the test compound mimicked the migration pattern of the ddNTP control.” Ex. 1016 (Metzker 1994) at 4263. Second, the substrate could lead to “inhibition.” Metzker explains that inhibition means “the presence of the test compound prevented the polymerase from incorporating the natural nucleotides.” *Id.* Finally, Metzker reported no activity “when the 3’-modified-dNTPs mimicked either the blank or readthrough controls.” *Id.*

64. In sum, Metzker 1994 distinguished between two different types of activity. Either a modified nucleotide prevented incorporation of natural dNTPs (“Inhibition”) or a modified nucleotide could be incorporated by a particular polymerase, resulting in the polymerase extension being stopped at a specific position (namely the same ones where synthesis was also terminated by a ddNTP) (“Termination”). Metzker 1994 assayed the modified nucleotides at concentrations that included 250  $\mu$ M. *Id.* In some instances, Metzker observed that the “activity” of a particular compound/polymerase compound (either inhibition or termination) “was incomplete” at the tested concentration. *Id.* Metzker labeled these “Inhibition\*” and “Termination\*.” *Id.*

65. Metzker 1994 explained: “In addition to termination, some readthrough was also observed due to the presence of contaminating dATP,” which

was present at approximately 1%. *Id.* In cases where Metzker carried out testing at 250  $\mu\text{M}$ , as with the 3'-O-allyl dATP, this means natural dATP was present at a concentration of approximately 2.5  $\mu\text{M}$ . As Metzker explains, this natural substrate competed with the non-natural substrate for incorporation, as evidenced by incorporation of the natural substrate and the production of a “readthrough” band. *Id.* This is shown, for example, in Figure 3B, which shows a consistent “readthrough” band for the 3'-O-methyl-dATP experiments. *Id.* at 4264

66. A skilled artisan would have appreciated that the presence of dATP contamination means that the tested compounds had to compete in Metzker’s assay with the natural substrate. Nonetheless, the 3'-O-allyl derivative was still able to terminate the reaction (i.e., the 3'-O-allyl derivative was still able to be incorporated, even in the presence of dATP). Thus, in my opinion, a skilled artisan would understand that any termination indicated that the modified dATP was a promising reagent for sequencing by synthesis.

67. Thus, even though the combination of certain 3'-O-modified dATP and certain polymerases did not have complete termination activity at a final concentration of 250  $\mu\text{M}$  (“Termination\*”), a skilled artisan would still understand, consistent with Metzker’s definition of “Termination,” that the modified dNTP could be incorporated (despite the presence of 2.5  $\mu\text{M}$  competing natural dATP) by the polymerase and act as a sequencing terminator. Specifically, a skilled artisan

would have understood that the 3'-O-allyl derivative could be used as a sequencing terminator in conjunction with the Vent(exo-) polymerase.

68. I understand that Columbia asserts that the meaning of “Termination\*,” as used in Metzker 1994, is that the substrate is “inefficiently” incorporated. This is not what Metzker teaches. Metzker never characterizes “Termination\*” as inefficient. Instead, Metzker indicates that the meaning of “Termination\*” is that termination is not completely achieved at a concentration of 250  $\mu$ M (and in the case of the dATP analogs, in the presence of approximately 2.5  $\mu$ M of the natural dATP). Ex. 1016 (Metzker 1994) at 4623. A skilled artisan would have understood that increasing the concentration of the 3'-O-allyl dNTP and/or increasing the reaction time, for example, would have been expected to result in more complete termination.

69. An artisan’s understanding of Metzker’s “incomplete” termination is further confirmed by Metzker’s use of the same language (“termination was incomplete”) when describing the use of *Pfu*(exo-) polymerase with ddNTPs in Table 1. Ex. 1016 (Metzker 1994) at 4262. As indicated in Table 1, an entry of I/T “means ddNTP termination was incomplete.” *Id.* Table 1 indicates that *Pfu*(exo-) has incomplete termination for ddNTPs. Ex. 1016 (Metzker 1994) at 4262. Contrary to Columbia’s position, this disclosure of incomplete termination does not mean that the ddNTP nucleotides are inefficiently incorporated by the *Pfu*(exo-) polymerase. To the contrary, a skilled artisan would have known that ddNTPs are efficiently

incorporated substrates, and *Pfu*(exo-) efficiently incorporated ddNTPs when the ddNTPs were provided at an appropriate concentration.

70. Before the year 2000, *Pfu*(exo-) was sold as part of a commercially available DNA Sanger sequencing kit, which used ddNTPs at a concentration of 450  $\mu$ M. Ex. 1108 (Stratagene Manual) at 1-2, 6, 15. As explained in the Stratagene Manual, “the presence of dideoxynucleotide triphosphates (ddNTPs) in the sequencing reactions . . . create[s] the base-specific terminations required for sequencing . . . .” *Id.* at 1.

71. The *Pfu*(exo-) sequencing procedure involves aliquoting 3  $\mu$ L of one ddNTP (concentration 1.5 mM) into 7  $\mu$ L of reaction mixture, for a final concentration of 450  $\mu$ M of the ddNTP when the sequencing is performed. *Id.* at 2, 6, 15. A skilled artisan would have understood that, in order to carry out Sanger sequencing using *Pfu*(exo-) with the commercial kit sold by Stratagene at the time, each ddNTP substrate would be reliably incorporated when the ddNTP was present at a concentration of 450  $\mu$ M.

72. A skilled artisan would have understood that, in order to carry out Sanger sequencing using *Pfu*(exo-) with the commercial kit sold by Stratagene at the time, these ddNTP substrates, when present at 450  $\mu$ M, would need to compete effectively with the natural dNTP substrate in order to reliably terminate at least some of sequences at every single base position. In other words, an artisan would



have understood that a requirement to carry out Sanger sequencing is that the ddNTP substrates need to be efficiently incorporated by the polymerase such that they could compete with the natural dNTPs present in solution for incorporation into the growing DNA strand. If the ddNTPs were not reliably incorporated in the presence of the competing natural substrate, the Sanger sequencing process would not be successful. This is because Sanger sequencing is premised on the incorporation of ddNTP into some growing strands at every position complementary to the ddNTP in the template strand. If the ddNTP were inefficiently incorporated, the natural dNTP would out-compete the ddNTP and no strands would be terminated, resulting in missing bands on the sequencing ladder, and errors in the determined sequence. Thus, in Sanger sequencing, substrates (e.g., ddNTPs) must be efficiently incorporated in order to compete with the natural dNTPs to terminate extension. In fact, if ddNTPs were only inefficiently incorporated, no bands would appear and the method would completely fail. This reliable efficiency requirement is even more pronounced in commercial kits, which are designed to provide reliable results without the need for optimizing conditions to accommodate the experimental requirements of the specific test being performed. Therefore, even though ddNTPs are used at a higher concentrations than dNTPs (*See* Ex. 1108 (Stratagene Manual) at 10, disclosing a 10x sequencing buffer containing 20-50  $\mu$ M dNTPs), a skilled

artisan would still understand that ddNTPs were efficiently incorporated by the *Pfu*(exo-) polymerase.

73. Thus, a skilled artisan reading Metzker 1994's statements regarding the "incomplete" termination of ddNTPs by *Pfu*(exo-) would have understood that "incomplete" termination is not the same as "inefficient" incorporation. Instead, in my opinion, a skilled artisan would have understood that Metzker 1994's disclosure of "incomplete" termination at a particular concentration of substrate indicates that a higher concentration of substrate would push the termination to completion.

74. This conclusion is particularly true when the reaction is carried out in the presence of the natural substrate, which competes with the modified dNTP for incorporation. Notably, the concentration of natural dNTP included in Metzker's *Pfu*(exo-) reactions (Ex. 1016 (Metzker 1994) at 4262 Table 1) were the same (2.5  $\mu$ M dNTPs) as the amount of contamination in Metzker's modified dATP reactions, including the 3'-O-allyl dATP reaction (approximately 2.5  $\mu$ M natural dATP). And Metzker varied the ddNTP concentrations in these experiments to concentrations as high as 250  $\mu$ M (Table 1, M-MuLV-RT). Thus, Metzker reports that, for the *Pfu*(exo-) enzyme, which was known to reliably and efficiently incorporate ddNTPs in a commercial kit, "termination was incomplete" under concentrations that appear to have been quite similar to those conditions used in Metzker's 3'-O-allyl dATP reaction (which also used 250  $\mu$ M 3'-O-allyl dATP and 2.5  $\mu$ M dATP). This

“termination was incomplete” therefore, is not synonymous with inefficiency. Instead, it is simply an indication that a concentration of 250  $\mu\text{M}$  was not enough to achieve complete termination in the amount of time that Metzker 1994 gave for its experiments. Those in the sequencing art knew that ddNTPs were good substrates for *Pfu*(exo-) polymerase at 450  $\mu\text{M}$ . A POSA reviewing the result for Metzker’s 3’-O-allyl nucleotide would have similarly had a reasonable expectation that increasing 3’-O-allyl nucleotide concentration (for example to 450  $\mu\text{M}$ ) could also result in its being efficiently incorporated and able to sufficiently terminate DNA extension. Table 1 of Metzker shows that when 3’-O-methyl dTTP incorporation with Vent(exo-) polymerase was tested, the dATP concentration was 0.5  $\mu\text{M}$ .

75. A skilled artisan would have understood that the concentration of the 3’-O-allyl dATP could be increased in view of Metzker 1994’s own disclosure that the concentration of a modified dNTP could be increased above the screening concentration of 250  $\mu\text{M}$ . For example, as shown in Figure 4B, modified dNTP terminators were used at concentrations of, e.g., 500  $\mu\text{M}$ , and demonstrated more intense termination bands with increasing concentration (Ex. 1016 at 4265 Fig. 4B). In other instances, Metzker 1994 included up to 10 mM of a 3’-O-modified dNTP. Ex. 1016 (Metzker 1994) at Figure 3A. 10 mM is 40 times the concentration used in Metzker’s screening reaction, 250  $\mu\text{M}$ , which was the highest concentration at which the 3’-O-allyl derivative was evaluated. Based on this disclosure, a skilled

artisan would have expected to be able to increase the concentration of the modified nucleotide to increase the degree of termination. *See also* Ex. 1016 (Metzker 1994) at Figure 4A (using 450 or 750  $\mu$ M of ddNTP terminators).

76. Finally, a skilled artisan would have appreciated that extending the reaction time would have likely increased the degree of incorporation, and termination, for a particular 3'-O-modified dNTP. This is underscored by the fact that Metzker 1994 appears to run the incorporation reactions for 10 minutes. Ex. 1016 (Metzker 1994) at 4262 (reaction time for Table 1 termination is 10 minutes).

77. As such, in my opinion, Metzker's disclosure of a particular modified nucleotide as leading to "Termination" or "Termination\*" would have been important to a skilled artisan, even if termination was incomplete at the tested concentration and reaction time. This is because the observation of any termination, particularly when termination occurs in the presence of competing natural dNTP, indicates that the non-natural substrate could, in fact, be efficiently incorporated by the particular polymerase. Moreover, a skilled artisan would have been impressed that the 3'-O-allyl dATP derivative was able to compete for incorporation with natural dATP. Thus, in my opinion, a skilled artisan would have considered that Metzker reported that the 3'-O-allyl nucleotide demonstrated termination activity—even if incomplete—and, thus, the 3'-O-allyl nucleotide was a promising terminator for SBS.

78. A skilled artisan in 2000 would have recognized that the panel of enzymes tested in Metzker 1994 is not limiting. An artisan would have been motivated by Metzker 1994's identification of activity for certain terminator/polymerase pairs to explore other polymerases. This would include polymerases within the same family, with known structural similarity, or even later-developed mutants of the polymerase identified by Metzker. In my opinion, it would have been a straightforward and routine step for a person skilled in the art to build upon Metzker's work by testing the identified blocked dNTPs having any observed termination in conjunction with a broader screen of polymerases.

79. In fact, numerous polymerases were known by 2000, and a skilled artisan would have appreciated that these polymerases, consistent with Metzker's results, had different substrate specificities and would thus be potentially useful for different modified substrates. As an example, a thermostable archaeal polymerase related to Vent was published in 1996, after the Metzker paper. Ex. 1133 (Southworth (PNAS vol. 93, pp. 5281-5285, May 1996)). Likewise, mutations to polymerases, meant to allow the polymerases to be more tolerant to nucleotides having unnatural substituents at the 3' positions, were developed after 1994. See Ex. 1130 (Tabor, U.S. 5,614,365); Ex. 1131 (Davis, U.S. 5,885,813); Ex. 1122 (Gardner Nucleic Acids Research, 1999, Vol. 27, No. 12, pp. 2545-2553.).

80. The polymerases Gardner reported would have been of particular interest to a skilled artisan in view of Metzker's work. Gardner was directed at identifying mutations that make the Vent polymerase more tolerant of different nucleotides, including nucleotides with unnatural substituents at the 3' position, specifically with mutations to the 488 and 499 positions of the polymerase, where alanine and tyrosine amino acids, respectively, are normally found. Ex. 1122 (Gardner) at 2549. It would have been a typical and routine step for a skilled artisan to test the 3'-O-modified dNTPs identified in Metzker 1994 as having at least some termination ability, including the 3'-O-allyl nucleotide, with other related enzymes or mutants thereof.

81. For example, given Metzker's report that the 3'-O-allyl protected nucleotides demonstrated termination with the Vent(exo-) polymerase, a person skilled in the art would have been motivated to screen related polymerases for 3'-O-allyl protected nucleotide termination activity. This would have included polymerases in the same family as Vent and mutants of the Vent polymerase, but it would have been a straightforward step to set up screens against additional polymerases as well.

82. In contrast to the straightforward disclosure of Metzker 1994, Columbia proposes a rather more complicated theory of what "Termination\*" means to a skilled artisan. In particular, Columbia identifies what it calls an "efficiency test

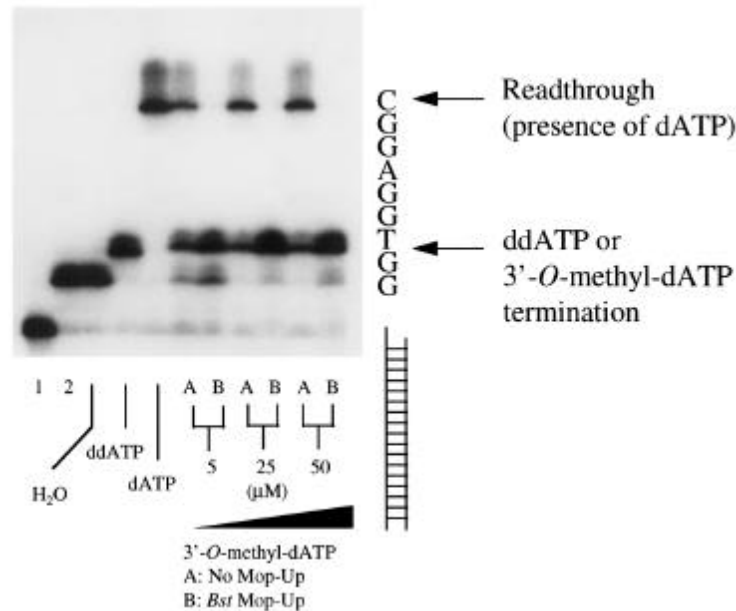
band” that Columbia asserts is indicative of inefficient termination. Metzker does not use the term “efficiency test band,” and this is not a term used in the art. Instead, Columbia made up this term for this proceeding, and Columbia made up a cartoon representation of what a gel for “Termination\*” would look like. Columbia argues that the presence of an “efficiency test band” in the made-up cartoon means the substrate is too inefficiently incorporated by the polymerase for SBS.

83. Dr. Menchen states that his basis for the cartoon representation of what a gel for “Termination\*” would look like was taken from a paper by Metzker published in 1998 (Ex. 2131). In 1998, Metzker published a follow-on paper that included a “mop-up” technique that removed the residual natural dATP present in the test samples in the 1994 paper. Ex. 2131 (Metzker 1998). This paper included no data for any 3’-O-allyl modified dNTP or any other experiment categorized as “Termination\*.” This paper included additional examination of the 3’-O-methyl-dATP, disclosed and identified as a terminator in the earlier 1994 work, as the test compound for the “mop-up” experiments.

84. As shown in Metzker 1998 Figure 3, reproduced below, the 3’-O-methyl-dATP substrate gave rise to a termination product when incorporated by the AMV-RT enzyme. Ex. 2131 (Metzker 1998) at 816. When the assay was run in the presence of natural dATP (“A” lanes), a readthrough band formed. When the assay was run without dATP (“B” lanes), no readthrough band was observed. Notably,

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the “A” and “B” lanes for 5  $\mu$ M 3'-O-methyl-dATP show a band at the same position as the “efficiency test band” in Columbia’s cartoon.



85. Metzker 1998 illustrates why Columbia’s understanding of “Termination\*” in Metzker 1994 is incorrect. Metzker 1994 and Metzker 1998 identified incorporation of the 3'-O-methyl-dATP with AMV-RT polymerase as leading to complete termination (not incomplete termination). Ex. 1016 (Metzker 1994) at 4263 (Table 2) and 4264 (Figure 3B); Ex. 2131 (Metzker 1998) at 816 (“Figure 3. Complete termination of 3'-O-methyl-dATP by AMV-RT.” and “Following enzymatic mop-up, the incorporation of 3'-O-methyl-dATP by AMV-RT shows complete termination without natural nucleotide read-through (Figure 3, lanes B).”). Therefore, both of Metzker’s disclosures show complete termination,



which under Columbia's theory means that the 3'-O-methyl-dATP is an efficient substrate of AMV-RT.

86. Yet, according to Dr. Menchen, he relied on Metzker 1998's data relating to this efficient substrate for understanding the meaning of "Termination\*." Metzker 1998 discloses that 5  $\mu$ M 3'-O-methyl-dATP reacted with AMV-RT, which shows an "efficiency test band" (for both lanes "A" and "B"). Columbia identifies such an "efficiency test band" as indicative of inefficient incorporation and incomplete termination. Yet there is no question that 3'-O-methyl-dATP was efficiently incorporated by AMV-RT. Ex. 1016 (Metzker 1994) at 4263 and 4264; Ex. 2131 (Metzker 1998) at 816. There is no way to square Columbia's assertion that this "efficiency test band" establishes that a compound is an inefficient polymerase substrate with the actual disclosures of both Metzker 1994 and Metzker 1998, which demonstrate that 3'-O-methyl-dATP is an efficient substrate of AMV-RT. Metzker 1998 shows that Columbia's "efficiency test band" goes away as substrate concentrations increase.

87. Metzker 1998 illustrates the role that substrate concentration has on the reaction. At low concentrations of the tested substrate, there is relatively more of the lower band ("efficiency test band"), reflecting that the 3'-O-methyl-dATP substrate is less incorporated, than at higher concentrations. Ex. 2131 (Metzker 1998) at 816. This is consistent with the expectation of a skilled artisan based on

the teachings of Metzker 1994 and 1998: as the concentration of the 3'-O-capped dNTP increases, the extent of incorporation increases, the resultant termination band increases, and the "efficiency test band" decreases. What a POSA would have understood from Metzker 1998 is that any modified dNTP would produce an "efficiency test band" at sufficiently low concentration, and that increasing the concentration results in efficient termination.

88. I understand that Columbia asserts that Metzker's "Termination\*" means that the 3'-O-allyl nucleotide showed that the nucleotide analog bound to the polymerase, but was slow to be incorporated into the DNA strand by the polymerase (in other words, Columbia asserts that the 3'-O-allyl-dATP binds and occupies the polymerase active site, but fails to be incorporated into the growing DNA strand), and thus it acts as an inhibitor. This is inconsistent with the termination seen. If 3'-O-capped nucleotides acted as an inhibitor, then increasing nucleotide concentration would result in increased inhibitor concentration, which in turn would result in any "efficiency test band" persisting at higher concentrations. However, Figure 3 of Metzker's 1998 publication shows that this is not the case. In Figure 3, the increased nucleotide concentrations resulted in efficient and complete termination. Exhibit 2131 (Metzker 1998) at 816 (Figure 3). Similar increased termination with increased nucleotide concentration is also seen in Metzker 1994,

Figure 4B. Ex. 1016 (Metzker) at 4265 (Figure 4B). Thus, Columbia's theory based on the "efficiency test band" in Columbia's cartoon drawing is incorrect.

89. Finally, Columbia downplays the amount of dNTP contamination observed in Metzker 1994. Yet Metzker 1998 recognizes that even the relatively low concentrations of dNTP contamination are "significant." Ex. 2131 (Metzker 1998) at 817. As discussed above, the amount of natural dNTP contamination in the product was substantial, approximately 2.5  $\mu$ M. Ex. 1016 (Metzker 1994) at 4263.

**Higher concentrations of modified dNTP could be used**

90. I understand that Dr. Menchen has taken the position that a skilled artisan would not have increased the concentration of a 3'-O-modified dNTP, specifically a 3'-O-allyl dNTP, above 250  $\mu$ M. As a result, I understand that Dr. Menchen believes that a skilled artisan would have avoided the otherwise routine step of increasing the concentration of a known substrate to increase nucleotide incorporation.

91. I believe that a skilled artisan would have considered it routine to increase the concentration of Metzker's modified nucleotide terminators to increase nucleotide incorporation. As discussed above, Metzker 1994 studied a wide range of modified nucleotide concentrations. See Ex. 1016 (Metzker 1994) at 4265 Fig. 4B (500  $\mu$ M 3'-O-modified NTP used); Fig. 4A (up to 5 mM of a 3'-O-modified dNTP used); *see also id.* (Fig. 4(A)) (using 450 or 750  $\mu$ M of ddNTP terminators);

Fig. 3A (up to 10 mM modified substrate). Based on this disclosure, a skilled artisan would have expected to be able to increase the concentration of the modified nucleotide to increase the degree of termination. Figure 4B of Metzker 1994 shows that efficient incorporation is achieved by increasing the concentration of 3'-O-dTTP from 20  $\mu$ M to 100  $\mu$ M to 500  $\mu$ M with Vent(exo-) polymerase. Ex. 1016 (Metzker 1994) at 4265.

92. I understand that Dr. Menchen believes that a skilled artisan would not have followed these teachings of Metzker 1994 based on the disclosure of another reference, Tsien (Ex. 1013). In particular, Dr. Menchen appears to have looked at Example 4 in Tsien as a definitive statement teaching a skilled artisan away from routine experimental changes. Ex. 1013 (Tsien) at 38. I do not consider that any teaching in Tsien would discourage routine experiments such as testing increased modified nucleotide concentrations.

93. The portion of Tsien identified by Dr. Menchen is, by its own terms, an "Example." Ex. 1013 (Tsien) at 38. Tsien specifically states that the examples are intended to be "nonlimiting." *Id.* at 9. Notably, in an earlier section discussing reaction conditions more generally, Tsien recognized that "to force the reaction, especially with derivatized dNTP's it may often be helpful to use substantial excesses (over stoichiometry) of the dNTP's," or modify other reaction conditions. Ex. 1013 (Tsien) at 20. This contradicts interpreting Tsien's Example of a

theoretical reaction as limiting the amount of dNTP, particularly in view of the later experimental work by Metzker using higher concentrations. Tsien's disclosure simply reflects the typical approach taken by a skilled artisan when seeking to drive a polymerase incorporation reaction to completion: increase the concentration of the nucleotide substrate.

94. Columbia, in footnote 14 of the Patent Owner's Response, quotes a portion of the Eckert article that states "high dNTP concentrations decrease polymerase fidelity by decreasing the amount of error discrimination at the extension step." Ex. 2125 (Eckert) at 21. As an initial point, Columbia does not explain what it means to be a "high" concentration of dNTP, and Eckert provides little specific guidance beyond a statement that "when the dNTP concentration is lowered from 800  $\mu$ M to 6  $\mu$ M, only T\*G mismatches or perfect base pairs are extended." Ex. 2125 (Eckert) at 21. The potential efficiency of misinsertion of a natural or modified dNTP, or extension of mismatch (such as T\*G) are experimentally tested on a case-by-case basis. In fact, as discussed above (see ¶¶65-75 above), several modified nucleotides were known to be accurately incorporated at concentrations greater than 250  $\mu$ M (the highest concentration tested for the 3'-O-allyl dATP derivative). For example, Stratagene's *Pfu* polymerase-based commercial sequencing kit used 450  $\mu$ M ddNTPs. Ex. 1108 (Stratagene Manual). Such a commercial kit would not be viable if 450  $\mu$ M concentrations were unduly prone to misincorporation.

95. In fact, the “errors” identified by Eckert are related to a polymerase’s sensitivity to substrate concentration. *See* Ex. 2125 (Eckert) at 21 (“Generally, the rate of polymerase extension from mispaired or misaligned primer-termini (Fig. 1, step D) is dependent upon the concentration of the next correct dNTP substrate.”). Eckert’s teaching relates to increasing natural dNTP concentrations beyond that required for efficient incorporation to concentrations that are far above the discrimination threshold of a polymerase to base-specifically incorporate dNTPs.

96. Eckert’s discussion is in the context of PCR using natural dNTPs. Eckert’s discussion does not include modified dNTPs that, while efficiently incorporated, have a larger  $K_m$  (weaker binding). This is why higher concentrations are used for accurate and efficient incorporation, but it will also likely increase the concentration required for misinsertion. The window between efficient correct incorporation and misincorporation is shifted to higher concentrations. A person working in the field of DNA sequencing would understand Eckert’s teachings indicate that the concentration required for misincorporation is higher than the concentration required for accurate and efficient incorporation.

97. A skilled artisan, while certainly aware of the concentration dependence of dNTP incorporation, would have looked to Metzker’s actual experimental results when considering the appropriate concentration of modified dNTPs. As reported in Metzker 1994, based on termination at the appropriate base,

the modified dNTP derivatives were correctly incorporated. Ex. 1016 (Metzker 1994) at 4263; *see also id.* Fig. 3A (using 1 mM, 5 mM, or 10 mM of 3'-O-methyl dATP).

**Sanger sequencing required efficient incorporation of chain terminators**

98. I understand that Dr. Menchen testified that the Sanger sequencing process did not have any requirement for terminator incorporation efficiency. I disagree. As I discuss above with respect to the commercially available Stratagene sequencing kit, efficient incorporation of chain terminators, specifically the ddNTPs used in Sanger sequencing, is an essential part of the Sanger sequencing process.

99. Sanger sequencing works by incorporating chain terminators, e.g., ddNTPs, into a growing DNA strand. Whenever this happens, it results in the termination of the strand's further extension. The key part of the Sanger sequencing process is that at least some ddNTP terminator must be incorporated at every base position of the strand of DNA being sequenced. This results in a "ladder" of DNA strands, terminated at every position that a particular nucleotide occurred in the template strand, which allows the sequence to be inferred. The failure to incorporate at least some of the terminator at any given position would lead to missed bases and misread sequences. Moreover, incorporation of the different ddNTPs must be relatively consistent, otherwise the intensity of the sequencing signal will vary and create problems with identifying the correct base at a particular position.

100. Sanger sequencing also requires that the natural substrate be incorporated into at least some of the growing DNA strands, otherwise the sequencing reaction would terminate immediately upon ddNTP incorporation and the DNA template could not be sequenced. Typically, this is not a problem because the natural substrate can compete effectively with the ddNTP terminators.

101. Sanger sequencing thus involves a balance between supplying ddNTPs at a sufficient concentration to compete with natural dNTP substrates for incorporation, while also ensuring that the resulting termination bands are consistent enough to provide useful sequencing information. An important point is that by simply increasing the concentration of the ddNTPs, their incorporation can be made to compete effectively with that of their natural dNTP counterparts while still being accurately incorporated.

102. As explained in the Tabor and Richardson patent (U.S. 5,614,365) (Ex. 1130), which used T7 polymerase, these are general requirements for Sanger sequencing:

Inherent to procedures currently used for DNA sequence analysis is the necessity to separate either radioactively or fluorescently-labelled DNA products by a gel permeation procedure such as polyacrylamide gel electrophoresis, and then detect their locations relative to one another along the axis of movement through the gel. The accuracy of this procedure is determined in part by the uniformity of the signal in bands which have permeated approximately the same distance through the gel. Differences or variations in signal intensities between nearby bands create several problems. First, they decrease the sensitivity of the method, which is limited by the ability to detect the bands containing



the weakest signals. Second, they create difficulties in determining whether a band with a weak signal is a true signal due to the incorporation of a chain terminating agent, or an artifact due to a pause site in the DNA where the polymerase has dissociated. Third, they decrease the accuracy in determining the DNA sequence between closely spaced bands since the strong signal of one band may mask the weak signal of its neighbor. See Tabor and Richardson, *supra*.

Variation in band intensity can arise from an inherent property of most DNA polymerases. Most DNA polymerases discriminate against the chain terminating dideoynucleotides used in DNA sequence analysis. T4 DNA polymerase discriminates against ddNTPs to such an extent that it cannot be used for DNA sequencing. (Ex. 1130 at col.2 1.55-col.3 1.13)

103. Sanger sequencing thus involved a balance of factors, including the need for a terminator to be incorporated with sufficient efficacy that it competes with the natural substrate. As such, I disagree with Dr. Menchen's position that a skilled artisan would not have understood Sanger sequencing to involve an efficient incorporation of terminators.

**A skilled artisan would have appreciated that different incorporation efficiencies were appropriate for different applications**

104. I understand that Columbia asserts that a skilled artisan would take a single view of incorporation efficiency without regard to the specific application. I disagree.

105. In my opinion, any criticism of different technologies or approaches must be understood in the context of the particular application of interest. When Columbia argues that different reversible terminators are inefficient substrates, it

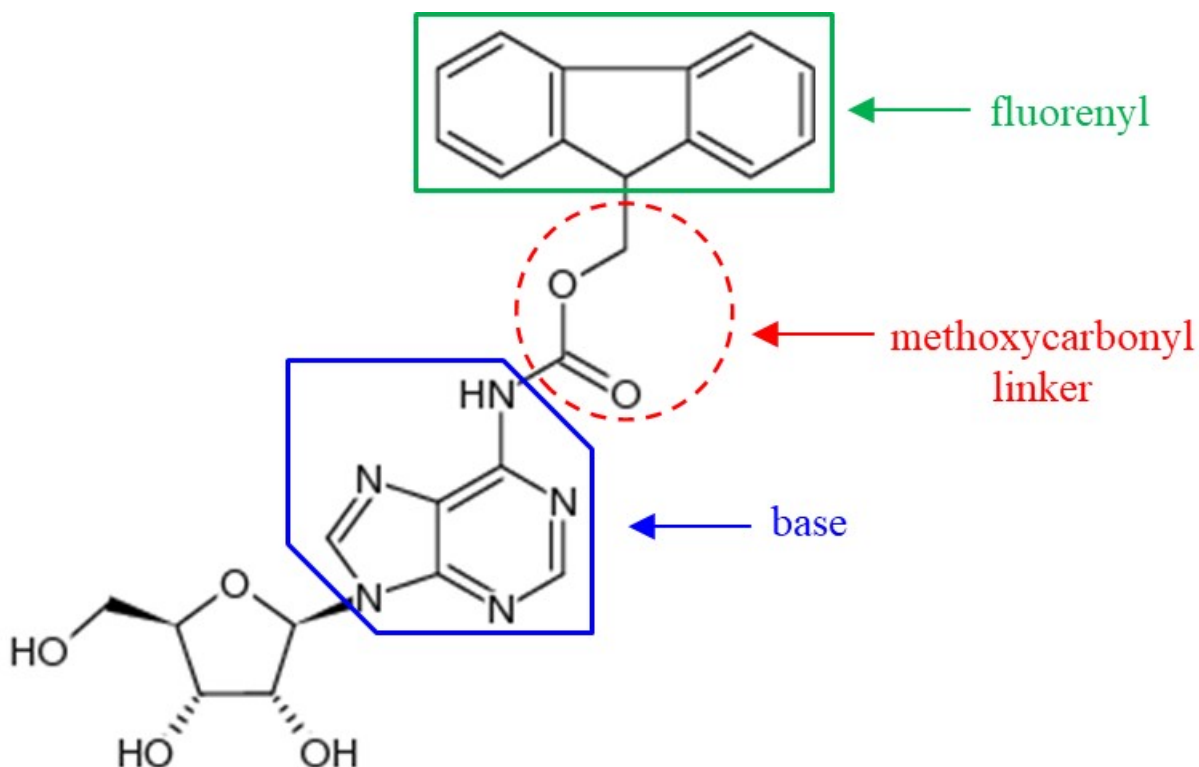
does not account for the particular requirements of different sequencing processes. For example, the Illumina sequencing process uses what Columbia might consider a relatively inefficient termination using modified dNTPs. The result is shorter sequencing read lengths. In contrast, other sequencing approaches, for example that used by Pacific Biosciences, use what Columbia might call more efficient incorporation, resulting in longer sequencing read lengths.

106. This efficiency, however, is only one factor in designing a successful sequencing system. I note that, despite what Columbia apparently believes is insufficient efficiency, the Illumina sequencing system is routinely used throughout the industry by commercial, medical, and academic organizations, including in my lab, and is more widely used than the method of Pacific Biosciences.

107. Ultimately, a skilled artisan would weigh the advantages and disadvantages of a particular approach in view of the overall needs of the system. Thus, a sequencing approach using a reversible terminator allows the creation of thousands of identical copies that can all be sequenced together. The ability to amplify a sequencing signal in this way makes the signal easier to detect and allows the use of less expensive instrumentation. In my opinion, the benefits of an SBS approach were well understood by skilled artisans at the time, as reflected by the interest of companies and researchers in the field.

### Dower's Fmoc Chemistry

108. Dower teaches labeling the base portion of a nucleotide analog by attaching a Fmoc (9-fluorenylmethoxycarbonyl) moiety to the nucleotide base. Ex. 1015 (Dower) at Fig. 9. The Fmoc moiety contains a cleavable methoxycarbonyl linker and a fluorescent fluorenyl dye. Ex. 1136 (Fields). Dower's Fmoc moiety connects the fluorescent fluorenyl dye to the nucleotide base via the cleavable methoxycarbonyl linker, as shown below:



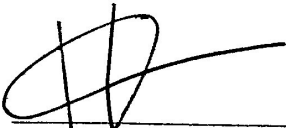
109. Fmoc was known to be cleaved at the methoxycarbonyl site, not at the fluorenyl moiety. As was known in the art, the methoxycarbonyl moiety that links the fluorescent fluorenyl ring structure to the nucleotide base is chemically cleavable under mild basic conditions:

The electron withdrawing fluorene ring system of the 9-fluorenylmethyloxycarbonyl (Fmoc) group renders the lone hydrogen on the  $\beta$ -carbon very acidic and, therefore, susceptible to removal by weak bases (1,2). Following the abstraction of this acidic proton at the 9-position of the fluorene ring system,  $\beta$ -elimination proceeds to give a highly reactive dibenzofulvene intermediate. (1-5). Dibenzofulvene can be trapped by excess amine cleavage agents to form stable adducts (1,2). (Ex. 1136 (Fields)).

110. The  $\beta$ -elimination cleavage produces carbon dioxide from the "oxycarbonyl" portion of the Fmoc. The ultimate products of the chemical cleavage are a free amine on the nucleotide base, carbon dioxide, and a modified fluorenylmethyl dye. Thus, in my opinion, the methyloxycarbonyl structure of the Fmoc group is a chemically cleavable chemical linker between the nucleotide and the fluorescent fluorenyl dye. I also note that a common use of an Fmoc group is to reversibly tag a molecule of interest with a fluorophore, and this is accomplished by attaching the fluorophore to the molecule of interest via a cleavable linker.

111. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Dated: Jan 22, 2019

By:   
Floyd Romesberg, Ph.D.