

## Transient Gene Expression: an Approach for Recombinant Vaccine Production

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### ABSTRACT

The production of recombinant vaccines in green plants is an attractive and promising topic in genetic engineering. However, the stable transformation of green plants is a time-consuming, costly, and labor-intensive practice. Moreover, public concerns about genetically modified plants put another limitation on the development and release of transgenic plant-based recombinant vaccines. These shortcomings were addressed by developing transient gene expression systems that allow researchers to investigate candidate recombinant vaccines quickly without tedious work and high costs. A comprehensive literature review was used to gather relevant information. This approach has received much attention in various recombinant vaccine production platforms, including mammalian cell culture, insect cell culture, yeast expression systems, and, more importantly, in plant hosts. Due to their simplicity and efficiency, transient gene expression systems are now widely used to validate gene constructs and transgene expression within plant tissues. This paper describes the concept of transient gene expression and discusses the significant advantages of this approach for producing recombinant vaccines. Notably, the major types of transient gene expression *viz.* agroinfiltration, viral-based systems, and application of naked plasmid in plant cell culture are introduced, and some examples illustrate the pros and cons of each system. Our literature review also discusses some practical notes on the successful application of this system to provide a more comprehensive image of transient gene expression applicability in green plants. As a whole, this review contributes to the existing literature by shedding more light on various aspects of transient gene expression that have not been addressed thoroughly yet.

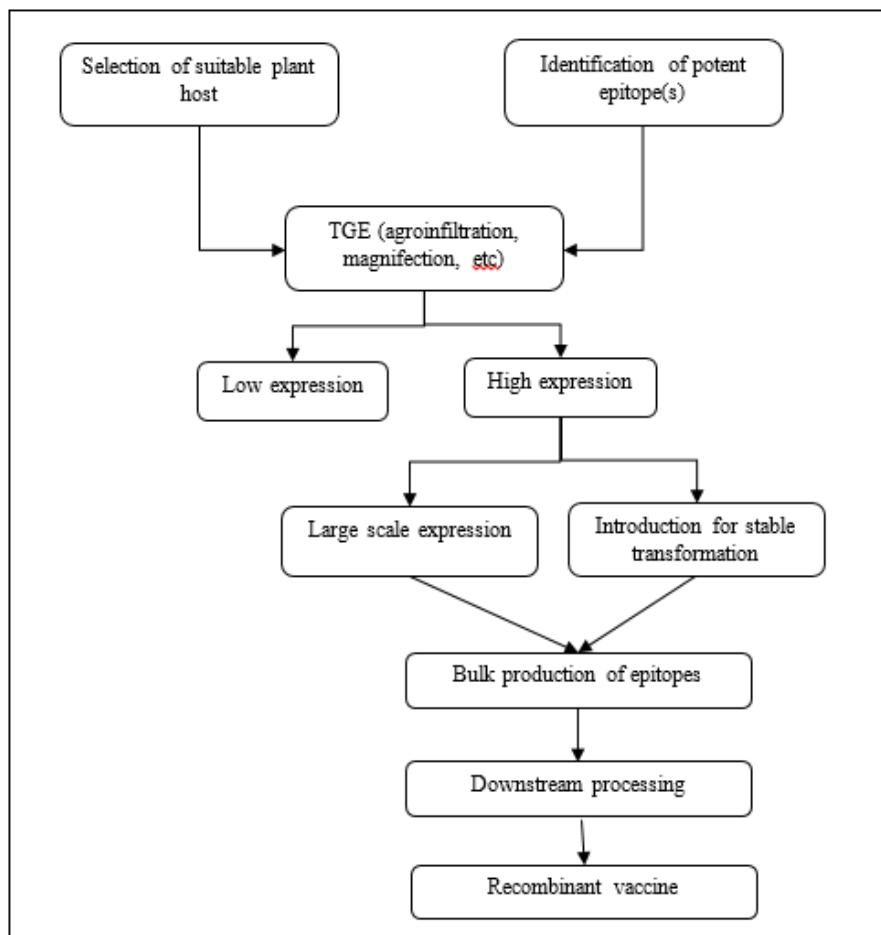
### INTRODUCTION

Many developed and developing countries have entered a contest to produce different recombinant vaccines in various plant species. Reviewing the literature shows that many scientific papers have been published during the current decade on the expression of viral epitopes in plant organs to develop edible recombinant vaccines [1]. Despite the large body of published papers, there have been really few examples of commercialized recombinant vaccines [2]. This apparent inconsistency implies that almost all the recombinant vaccine production efforts are limited to laboratory trials and still far from commercialization. Some factors account for this situation. First, despite the theoretical advantages of plant-based recombinant vaccines, such pharmaceuticals production through plants' genetic transformation would take a long time. Preclinical and field trials of candidate vaccines require high costs,

which are not affordable for many research teams, especially in developing countries. Regulatory issues and public concerns about genetically modified materials put further hurdles on the way toward the commercialization of recombinant vaccines [3]. Based on these and other considerations, many researchers have adopted transient gene expression (TGE) systems as an alternative for stable transformation to validate gene constructs designed to develop recombinant vaccines. TGE approach significantly shortens the period required for the evaluation of gene constructs [4]. Indeed, all molecular assays conducted in stable transformation for evaluating transcription and translation of bacterial or viral epitopes in plant tissues can be performed through TGE experiments [5]. Moreover, TGE systems are simple and easy to perform and provide a significant cost saving.

These properties make TGE an ideal platform for developing and validating recombinant vaccines at the laboratory level [6]. A schematic illustration of recombinant vaccine production using the TGE approach

is represented in Fig 1. As seen, TGE can be used either independently or as a complementary step for stable genetic transformation toward the development of recombinant vaccines.



**Fig 1.** Successive steps for the development of recombinant vaccines using transient gene expression approach

This paper reviews various TGE systems developed to express target antigens and the production of recombinant vaccines. The advantages and disadvantages of each system are discussed and illustrated by some examples. The paper is concluded by a comprehensive discussion on the necessity, pros, and shortcomings of TGE systems to develop plant-based recombinant vaccines.

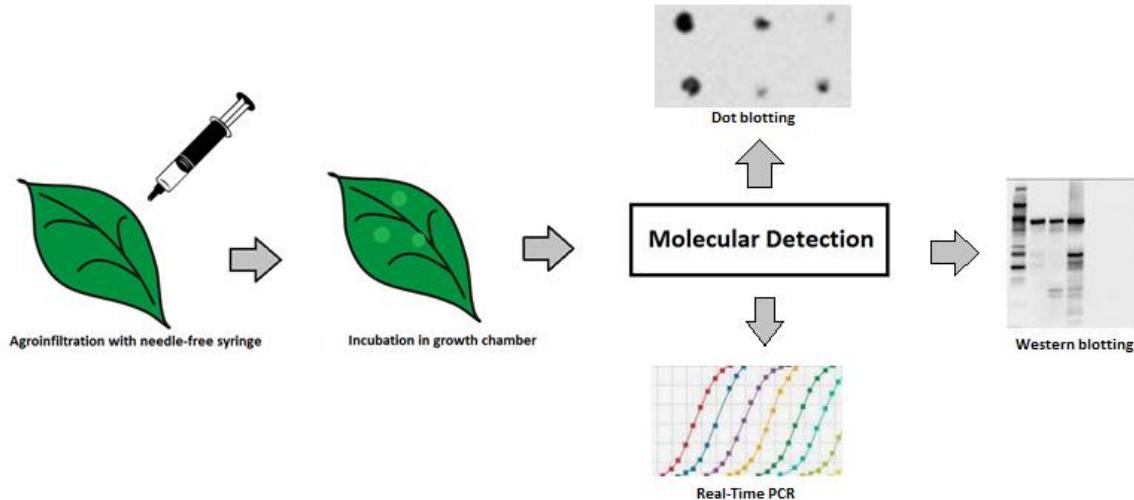
### Transient gene expression

In the standard procedure for the production of recombinant vaccines -the so-called stable transformation - target epitopes or antigens are integrated into the plant genome, and production of the recombinant vaccine is achieved by seedling regeneration. This approach requires tissue culture techniques that are considerably time and cost-consuming and prone to contamination with plant pathogens [7]. Transient gene expression (TGE) systems

have been developed to address the problems of stable transformation. In this approach, the target antigen encoding gene could not be integrated into the plant genome but resides in plant cells and directs the recombinant protein expression. This strategy offers many advantages like significant shortening of the time frame for producing recombinant vaccine, e.g., from two years to one week [8]. Expression of antigen in plant tissue can be achieved by significantly lower cost and efforts. Simultaneously, recombinant protein accumulation is significantly enhanced due to addressing the negative position effect caused by random integration of transgene in plant genome during stable transformation [9]. Also, stable transformation usually depends on *Agrobacterium*-mediated gene transfer, limiting plant hosts to dicotyledons since monocotyledons are not naturally infected by *Agrobacterium tumefaciens*.

In contrast, TGE systems are applicable to monocotyledon and dicotyledon plants, significantly broadening the range of plant species used to develop recombinant vaccines [5]. Moreover, *in vivo* assays have provided persuasive evidence that recombinant vaccines produced through TGE systems have sufficient potency

to combat diseases. For example, Hong *et al.* (2019) reported TGE-based development of H1-ss-np as a promising candidate for a universal influenza vaccine [10].



**Fig 2.** Schematic presentation of agroinfiltration procedure

Similarly, Beihaghi *et al.* (2018) found that recombinant CCL21 chemokine produced by TGE in tomato had a potent anti-metastatic function when used under *in vivo* conditions [11]. Mardanova *et al.* (2019) reported that recombinant influenza A vaccines produced in TGE plant tissues could stimulate broad-range protective immunity [12]. More examples of the efficacy of TGE-based recombinant vaccines in the induction of immunological response in animal hosts have been provided by [13].

Table 1 represents a brief comparison between stable transformation and transient gene expression systems. As can be seen, TGE offers various advantages over stable transformation. TGE systems present numerous advantages such as simplicity and time, and cost-saving, and do not need complex procedures nor expensive devices. These properties render TGE an appropriate and efficient system for investigating various antigens and epitopes nominated to develop recombinant vaccines [14]. During the last two decades, different TGE systems have been developed and validated to produce pharmaceutical proteins and peptides such as monoclonal antibodies, growth hormone, and recombinant vaccines [15]. In the following sections, we introduce various TGE systems and show their applicability for transient expression of recombinant vaccines in plant cells.

### Agroinfiltration

Agroinfiltration represents one of the simplest TGE methods and is widely used by researchers for transient expression of the target antigen in plant leaves. This method includes injection of *Agrobacterium* suspension carrying the transgene into plant leaves [16]. Agroinfiltration may be performed by either syringe infiltration or vacuum infiltration. The former includes injecting *Agrobacterium* suspension into plant leaves (or other organs) using a needle-free syringe, while the latter is performed by putting the leaves and bacterial suspension in a vacuum chamber [8].

Due to its simplicity and quick nature, agroinfiltration has been used for the production of recombinant vaccines. For example, Lombardi *et al.* (2009) investigated the applicability of agroinfiltration for the development of anti-HIV vaccines and obtained high levels of HIV-1 Nef expression when the gene construct was transiently expressed in tobacco (*Nicotiana benthamiana*) leaves using agroinfiltration [17]. He *et al.* (2012) used agroinfiltration for rapid and cost-effective production of recombinant vaccines against the West Nile virus and concluded that this procedure could be scaled up for commercial production of recombinant vaccines [18]. Matsuda *et al.* (2018) investigated the production of recombinant hemagglutinin (anti-influenza epitope) in leaves of *N. benthamiana* using agroinfiltration and concluded that the approach is quite

efficient for large scale production of recombinant epitopes [19]. Beihaghi *et al.* (2018) evaluated the expression of recombinant protein of C-C chemokine ligand 21 (CCL21) in *Solanum lycopersicum* via agroinfiltration and obtained high levels of recombinant protein expression three days after infiltration [11]. Researchers at the Canadian biotechnology company Medicago used agroinfiltration to produce the influenza vaccine in *N. benthamiana* leaves. According to the reports, this procedure allows for the bulk production of

influenza vaccines in a reasonably short time [20]. Habibi-Pirkoohi *et al.* (2014) expressed VP1 epitopes of foot and mouth disease virus (FMDV) in tobacco (*N. tabacum*) and observed high levels of recombinant peptide accumulation in infiltrated leaves (Fig. 3a). They also examined the agroinfiltration technique to express the same construct in spinach [8] and alfalfa [21]. High levels of antigen expression were achieved in both experiments.



**Fig 3.** Agroinfiltration through syringe infiltration of *N. tabacum* leaves (Habibi-Pirkoohi *et al.* 2014)

As mentioned before, agroinfiltration can be conducted by vacuum infiltration technique. In this procedure, seedlings or plant tissues are submerged in *Agrobacterium* suspension under vacuum conditions provided by a vacuum pump [7]. Chen *et al.* (2016) applied this technique for transient transgene expression in lettuce seedlings [22]. Mohammadzadeh *et al.* (2014) expressed hepatitis C virus core protein in potato leaves using vacuum infiltration and stated that this technique allows for high levels of transient antigen expression in plant leaves [16].

The main advantages of agroinfiltration include simplicity and applicability in various fields. Leaf agroinfiltration with a simple syringe does not require sophisticated skills and is conducted in a short time [23]. The same advantage is also the case for vacuum infiltration, which can be completed in a reasonably short period [24]. Moreover, agroinfiltration has been proved feasible in different experiments, including biochemical characterization, purification and preclinical functional studies, plant-pathogen interactions, abiotic stresses, and plant gene function analysis well as others [22]. In contrast to conventional *Agrobacterium*-based permanent genetic engineering approaches, agroinfiltration is not limited to dicotyledon plants which extend its use in a more significant number of plant hosts [25].

#### Agroinfiltration in the form of magnification

Despite their significance in producing recombinant subunit vaccines, viral systems suffer from limitations in foreign gene size. Only foreign genes with a certain length can be successfully inserted into viral genomes and expressed in plant tissues. Moreover, the limited host range of viruses will restrict the applicability of this system in many plant species [14]. To address this drawback, Gleba *et al.* (2005) integrated the advantages of *Agrobacterium* and viral vectors for TGE assays and developed a new system called magnification for enhancing the expression level of transgenes in plant tissues. In this technique, an *Agrobacterium* carrying a viral replicon is used for infiltration of plant tissues. According to the authors, "This improved process is being used to simultaneously start transient gene amplification and high-level expression in all mature leaves of a plant, and such a transfection can be done on an industrial scale" [26]. Based on this system, the *Agrobacterium* plasmid addresses foreign gene size because long gene fragments can be inserted successfully in the bacterial plasmid. Simultaneously, the viral replicon integrated into the vector allows for rapid propagation of the plasmid within plant tissue. This approach results in a considerable accumulation of the recombinant protein in plant hosts [27].

Using magnification, Santi *et al.* (2008) successfully expressed Norwalk virus-like particles (VLPs) in *N. benthamiana* leaves and observed that the transgene was expressed at 0.8 mg/g leaf less than two weeks after

infection. Moreover, the VLPs were able to induce systemic and mucosal immune responses in mice [28]. Zelada *et al.* (2006) used magnification to express tuberculosis antigen ESAT-6 in *N. tabacum* [29]. Fahad *et al.* (2015) referred to magnification as a robust and novel approach for therapeutic protein expression in plants [27]. Similarly, Muneerappa *et al.* (2018) expressed that magnification is an effective technology for developing recombinant oral vaccines in plant hosts, enabling the production of large quantities of foreign epitopes within short time frames [30]. Considering the significant advantages and unique properties of magnification, it can be postulated that this technique combines the pros of viral-based and *Agrobacterium*-based TGE systems and will be the choice of many researchers to develop various types of recombinant

vaccines. The main advantage of magnification is the ability of this system to transmit large gene fragments that allow the production of recombinant vaccines with large epitopes. This feature plays a vital role in facilitating the design process of structures related to recombinant vaccines and proteins. Simultaneous use of *Agrobacterium*'s innate ability to deliver large fragments into plant hosts, together with viral replicons for rapid proliferation within plant tissues, has given magnification a unique capability that distinguishes it from other transient expression systems [29]. However, the number of plants that have been transformed so far is low, and laboratory systems still need to be optimized to transform more species. In addition, viral replicons may not reproduce well in all plant species, thus reducing gene expression efficiency [31].

**Table 1.** Comparison between stable transformation and transient gene expression systems for the development of recombinant vaccines

Expression type	stable transformation	transient gene expression
Required time	Long	Short
Required cost	High	Low
Gene escaping risk	Yes	No
Expression level	Low	High
Sophisticated devices	Required	Not required
Plant host range	Limited	Fairly wide
Tissue culture procedure	Always required	Not required in most cases

**Table 2.** Pros and cons of various transient gene expression systems for the development of recombinant vaccines

Expression type	Advantages	Limitations
Agroinfiltration	No need for tissue culture, large transgene size, quick-expression, low cost, applicability in monocots	Infeasibility in some organs such as roots, waxy leaves, and woody tissues
Viral vectors	high level of recombinant vaccines production in plant tissue, straightforward treatment of plant tissues, quick transmission within whole the plant	A relatively narrow host range, limitation in transgene size
Plant cell culture	analysis of protein stability and activity; possibility to analyze a large number of samples in a short period, convenience, and flexibility	High cost and complex process of cell suspension production, technical difficulty in maintenance of cell suspension

### Viral vector-based transient gene expression

Plant viruses have been widely used since the mid-1980s for transient expression of proteins in plant tissues. Indeed, viral vectors have contributed more than *Agrobacterium*-based techniques to the transient expression of plants' target genes [32]. The widespread use of viral vectors for the expression of recombinant therapeutics in plants stems from the fact that viruses produce high copy numbers in really short times, resulting in high levels of recombinant protein accumulation. Thus, this technique is considered an efficient, rapid, and scalable procedure for the development of recombinant vaccines [33]. In a broad categorization, recombinant vaccine production using viral vectors can be realized by two approaches: 1- the fusion of antigen to virus coat protein (epitope presentation); 2- the expression of whole foreign antigen individually or as part of a fusion protein (polypeptide expression system) [14]. Tobacco mosaic virus (TMV), cowpea mosaic virus (CPMV), alfalfa mosaic virus (AMV), and potato virus X (PVX) are the main viruses

widely used for the development of recombinant vaccines and other biopharmaceutics in plant tissues using the TGE approach [34-37].

Examples of recombinant vaccine production using viral vectors are numerous. Wigdorovitz *et al.* (1999) used viral vectors to express the whole VP1 coat protein of FMDV in alfalfa and reported that the recombinant vaccine was accumulated at high levels in alfalfa leaves [37]. Chung *et al.* (2011) expressed a chimeric protein consisting of human hepatitis A virus VP1 and an Fc antibody fragment in *N. tabacum* using viral vectors, and the recombinant antigen purified from the infected leaves was able to induce an immune response in the animal model [33]. Saejung *et al.* (2007) used tobacco mosaic virus (TMV)-based transient expression for expression of dengue 2 envelope protein in *N. benthamiana* and reported high expression of the epitopes in plant tissues [38]. In a similar work, Marques *et al.* (2014) expressed dengue 2 envelope domain III in cowpea plants using Cowpea Mosaic Virus (CPMV) system and reported that using this system allows the recombinant protein to

retain its immunogenic properties [39]. Golovkin *et al.* (2007) reported that the smallpox subunit vaccine produced in plants using a viral vector system protected the animal models against artificial challenges [40].

Reviewing various systems for molecular pharming in plant hosts, Ibrahim *et al.* (2019) suggested that viral vectors are efficient TGE systems for developing recombinant pharmaceutical proteins. A high expression rate in a reasonably short time was regarded as the main advantage of viral vectors. The resulting recombinant vaccine was able to induce an immunogenic response in the animal model [41]. These and numerous other examples show that viral vectors are ideal tools for transient expression of recombinant vaccines in plants. It seems that advancements in molecular biology and elucidation of more facts about plant viruses' genetic apparatus will lead to further achievements in the future. High levels of transgene expression and consequently high production levels of recombinant vaccines in plant tissue is one of the essential advantages of viral vectors [33].

On the other hand, treating host plants with viral vectors is not a complicated task and can be done quickly. Rapid transmission of the virus in the host plant's vegetative tissues is another feature that makes the viral vectors a desirable vector for the production of recombinant vaccines [32]. However, the narrow range of plants that host current viral vectors is one of the disadvantages of viral vectors in transient gene expression. Another limitation of viral-based transient gene expression is the virus' low infectivity in some plant species and its inability to carry average-size or larger transgenes [40].

### Application of naked plasmid

Cell and protoplast cultures represent another platform for efficient transient gene expression. In one of the first attempts, Dekeyser *et al.* (1990) successfully expressed  $\beta$ -glucuronidase (GUS) in rice cell culture using electroporation [42]. Yoshioka *et al.* (1996) used *Agrobacterium*-based TGE for expression of  $\beta$ -glucuronidase (GUS) in tobacco cell cultures and observed the maximum expression level 48h after co-culture, concluding that TGE can be used as an efficient and rapid approach for expression of foreign genes in plant tissues [43]. More than a decade ago, Vermij and Waltz (2006) expressed hemagglutinin-neuraminidase in tobacco suspension cultures. This work was a milestone in developing recombinant vaccines in plant cells since the resulting vaccine received USDA's Center for Veterinary Biologics approval to be released as a Newcastle Disease vaccine [44]. In a review article, Takeyama *et al.* (2015) discussed various advantages of transient gene expression for developing recombinant vaccines against human and livestock diseases [45]. In general, cell cultures' main advantages for developing recombinant vaccines include quick identification of

suitable cell lines, containment nature that prevents horizontal gene transfer, and reproducibility [46].

Transient expression of genes in plant protoplasts is a powerful tool in recombinant vaccine production and plant molecular biology that allows rapid and convenient analysis of recombinant proteins before the stable transformation. Despite various advantages of the stable transformation of plant species in molecular farming, plants' transformation and regeneration are time-consuming, tedious, and require plenty of space [47]. Transient expression in cell and protoplast cultures enables measurement of gene expression, as well as analysis of protein stability and activity, very shortly after DNA uptake; which allows for screening a large number of samples in a fairly short period of time [48]. Moreover, most of the introduced plasmid DNA remains extra-chromosomal during the transient assay, avoiding gene activity being biased by position effects, as observed in stable transformation assays. Due to various advantages of transient gene expression such as speed, convenience, and flexibility, it has been widely used for the analysis of promoter and regulatory elements involved in transcription and translation, induction of gene expression by exogenous stimuli, and for verifying the functionality of cloned genes or cDNAs [49].

### Concluding remarks

According to the current opinion, the technology of transient gene expression in plant systems has considerable advantages over the stable expression, namely, (i) it does not require regeneration of the transformed cell; (ii) does not affect the stability of the host genome; and (iii) is independent of the position effects of the T-DNA integration sites.

Compared to stable transformation, TGE approaches offer several advantages, including simplicity [3, 5], time- and cost-saving [6], no need for expensive and complicated devices and procedures [44], lack of position effect, and gene silencing [49], and high levels of transgene expression [29, 33], among others. Moreover, the TGE system extends the range of plant hosts to produce recombinant vaccines and other proteins of pharmaceutical value [1]. Agroinfiltration is the simplest and the quickest TGE system, widely used by researchers to evaluate a target gene expression in plant tissues. Theoretically, this technique can be used in all plants, and there is no limitation in the choice of plant species for the expression of antigens. Tobacco plants, especially *N. benthamiana*, have been the most common agroinfiltration targets [17, 50]. However, edible plants such as lettuce [22], spinach [8], and potato [15] have also been treated by agroinfiltration. Viral vectors are the most widely used tools for transient expression of antigens and epitopes in plants since the viral particles are propagated quickly within plant tissues and provide high accumulation rates of the transgenes [14]. Empirical

evidence implies that viral vectors are suitable for producing considerable amounts of recombinant antigens within a short period [36]. However, the number of plant species that are hosts to viruses is limited, which is a significant disadvantage of the viral vector-based TGE system. Moreover, when foreign gene size exceeds a particular value, viral vectors' infection rates diminish significantly [16].

Magnifection is a vector system that uses an agroinfiltration technique for transient expression of recombinant peptides in plant tissues that combines the advantages of both *Agrobacterium*-mediated and viral vector-based systems [27]. Studies aiming to produce recombinant subunit vaccines have approved that magnifection is an efficient and affordable TGE tool [26-27]. In general, every TGE system possesses its pros and cons, and no single system can be deemed superior over the others. The choice of the TGE system may depend on experimental conditions and objectives. It is expected that the continued advancement of genetic engineering tools will lead to significant improvements in the production of subunit vaccines based on TGE systems.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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