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Method development for the analysis of PBMC-mediated killing of K562 cells by bovine colostrum

Anna Andersen *, Paula L. Vieira-Brock, Brent Vaughan, David Vollmer

4Life Research, 9850 S. 300 W., Sandy, UT 84070, USA

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ABSTRACT

Background: Colostrum is the first milk that supplies newborns with immune supporting peptides. Due to its heterogeneous and variable characteristics, standardized assays for assessment of its biological activities are a challenge. The current set of studies were aimed to investigate the immune activity of bovine colostrum blends as well as develop a method to assess variability across different lots.

Methods: Immune activity of a bovine colostrum blend was evaluated by their ability to enhance PBMC (peripheral blood mononuclear cell)-mediated killing of K562 cells. K562 cell killing was assessed by flow cytometry using DAPI. PBMCs derived from multiple healthy donors were initially investigated. Frozen PBMC aliquots from one of the highest responders were used for subsequent studies. Different doses and lots of product were assessed. Incubation time was also explored.

Results: Bovine colostrum blend similarly reduced K562 cells number and these effects were often greater than the IL-2 positive control. Despite consistent efficacy at enhancing PBMCs-mediated K562 killing, the degree of the effect was significantly variable across different lots. These biological effects were largely dependent on the solubility of the product.

Conclusion: Assessment of PBMC-mediated killing of K562 cells by flow cytometry using DAPI can be a reliable method for measuring immune activity of bovine colostrum when the material is well-dissolved into solution and the same biological sample from a single donor is used.

1. Introduction

There are several ways to support the human immune system: non-supplemental such as hand washing, and hygiene, nutrition (Calder et al., 2020), pharmaceutical ways such as vaccines and their adjuvants (Bastola et al., 2017), immunodrugs; including dietary supplements and other natural products such as colostrum and botanicals (Patwardhan and Gautam, 2005).

Assessing immunomodulation in *ex vivo* assays using PBMCs has many challenges. Some examples include assay limitations, such as variability in PBMCs from different blood donors; and sample-related challenges such as natural variability between batches of similar products that are botanical or proteinaceous by nature and the inherent solubility of such items being tested.

Such a method can be used in a number of contexts. Many researchers and privately-owned companies study different compounds' effect on the immune system. One of the purposes for which this method was developed was to create a way to directly measure an ingredient's effect on key parts of the immune system and to help standardize its use. Analytical methods exist to measure actives such as vitamins and minerals, but few exist to measure biological activity of natural compounds pertaining to NK (natural killer) cells (Wullner et al., 2010). The main ingredient this method was designed to test is bovine colostrum and its various fractions.

Colostrum is the first milk that supplies newborns with immune-supporting peptides. Colostrum contains a large variety of components including fats, proteins and peptides, oligosaccharides and other carbohydrates, vitamins, minerals, and more. As mentioned earlier, due to its complex, heterogeneous, and variable characteristics, standardized assays for assessment of its biological activities are a challenge. Examples exist in the literature of research showing that some of the immunologically active substances in colostrum such as immunoglobulins (Jasion and Burnett, 2015) and transfer factors (Li et al., 2010) survive the digestive tract upon oral ingestion and are absorbed into the bloodstream with their biological activity intact. Therefore it is appropriate to test effects of these and other immunologically active materials

* Corresponding author.
E-mail addresses: annaa@4life.com (A. Andersen), paulab@4life.com (P.L. Vieira-Brock), brentv@4life.com (B. Vaughan), davidv@4life.com (D. Vollmer).

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in vitro first, as a quick and cost-effective screening measure before testing *in vivo*.

Few methods exist to measure activity of NK cells after stimulus with complex biological actives. Flow cytometry is an effective method for evaluating the activity of NK cells (Kandarian et al., 2017). Previous studies in the literature have shown flow cytometry to be a useful method to test many kinds of biological activity in compounds before introduction *in vivo* (*e.g.*, Ostafe et al., 2013). The current set of studies was also aimed to use flow cytometry as a screening method to investigate the immune activity of bovine colostrum blends as well as to develop a method to assess variability across different lots of colostrum. This study is the first step in determining biological activity in this compound. The next step is then to investigate potential effects when introduced to a living organism.

2. Methods

Immune activity of a bovine colostrum blend, hereafter "compounds", was evaluated by its ability to enhance PBMC-mediated killing of K562 cells, a commonly used leukemia cell line. Interleukin-2 was used as a positive treatment control.

Frozen PBMC were thawed, washed, and counted, then diluted in RPMI medium containing 10% HI FBS (heat-inactivated fetal bovine serum), then were plated in a 96 well plate at a concentration of 96,000/90uL and allowed to "recover" for 16 h. After thawing, they were evaluated for CD56 expression using DAPI, with an approximate average of 12.6% of PBMCs expressing as CD56 during the first four rounds of testing (CD56 rates varied between 7.80% and 16.56%). Cell viability rate of PBMCs was measured and averaged over 95% throughout the experiment.

A number of compounds were provided in powder form from 4Life Research. These compounds consisted of defatted, pasteurized, ultrafiltered bovine colostrum. The compounds were solubilized to 10 mg/mL in PBS (phosphate-buffered saline) pH 7.4, 50 mM citric acid solution, or 40:60 citric acid solution/PBS. Compounds were mixed with solvent, sonicated at room temperature until dissolved/suspended, or for up to one hour. Compounds in solution were added to PBMC for 24 h yielding a final concentration of 1000μg/mL. K562 cells were then added into wells at 4000 per 90uL (20:1 Effector to target ratio) and incubated at different time points up to 48 h. Controls for this study included PBMC alone, PBMC + K562, and PBMC + K 562 + IL-2 (20 ng/mL). 4Life also provided an internal control (Colostrum Control) consisting of a composite of 11 lots of colostrum filtrate, which was included in all runs. All samples and controls were run in triplicate. After incubation, plates were spun at 1500 rpm, the supernatant removed, and the cells resuspended in PBS plus DiO. K562 cell killing was assessed by flow cytometry. Various incubation times and doses were explored, as well as lot-to-lot variability for 11 batches of bovine colostrum harvested from different dairies and different seasons of the year. These 11 lots of the colostrum ingredient were composited to create the Colostrum Control. Each of these lots may have been pooled from several different dairies prior to processing. Different PBMC donors were also compared.

2.1. Time and dose responses

Three doses and three incubation times were all tested in one run in order to determine the best parameters for future testing. 6-h, 24-h, and 48-h incubation times were all tested on one compound ("Colostrum Control"), alongside the IL-2 positive control and a negative solvent control. Three different concentrations of this compound were also tested: 10 μg/mL, 100 μg/mL, and 1000 μg/mL. The solvent for these samples was PBS. The IL-2 control was tested at once concentration, 20 ng/mL, at all three incubation time periods.

2.2. Donors' responses to controls

The next experiment was designed to determine how much variability could be due to differences in PBMC response to stimulus. PBMC were harvested from healthy blood donors. Six donors were tested with both the Colostrum Control at 1000 μg/mL and IL-2.

2.3. Lot-to-lot variability

The 11 different lots of the colostrum ingredient that had gone into the Colostrum Control composite were tested individually for activity. Initially they were tested with PBS as the solvent. After performing the solubility experiment (described below), they were re-tested in 50 mM citric acid in Part II.

2.4. Solubility experiment

Initially, compounds were tested using PBS pH 7.4 as the sample solvent, though different solvent systems were explored in subsequent rounds of testing. In one phase of the experiment, the colostrum control was tested side-by-side in both PBS and a 50 mM citric acid solution. In a separate experiment, 100 mg of sample was sonicated in 50 mM citric acid solution, after which PBS was added slowly with further sonication to QS to 10 mL. Different ratios of citric acid solution to PBS were used.

2.5. Data analysis

Differences between groups was explored using ANOVA. Statistical significance was determined with the *t*-test method. Results were considered statistically different if $p < 0.05$.

3. Results

3.1. Time and dose responses

Shown in Fig. 1 are the results of experimenting with different concentrations and time points, expressed as K562 events per microliter. Included are the Colostrum Control and the IL-2 control. The negative solvent control, showing NK cell response to K562 without any outside stimulus, is also shown. Further rounds of testing used all three controls for comparison purposes. An increasing response corresponding with increasingly longer incubation time was observed for all three controls at all three time points, as well as a dose-response for the three doses of the Colostrum Control, when compared to each other.

At 6 and 24 h, there was no significant difference in response between the negative solvent control and the positive IL-2 control ($p = 0.7$ and $p = 0.5$, respectively). The 48-h time-point did show a difference, with a lower K562 cell count on the positive IL-2 control ($p = 0.01$). Looking at the three doses of the Colostrum Control, the 1000 μg/mL dose was statistically different from the negative solvent control at all three time points. The 100 μg/mL dose was only significantly different than the negative solvent control at the 24-h time-point ($p = 0.004$), and the 10 μg/mL dose was significantly different from the negative solvent control at the 24-h and 6-h time-points ($p = 0.001$ and $p = 0.03$, respectively). Comparing the three doses to each other, a clear dose-response was observed for all three time-points, indicating that the best and most significant NK cell response was obtained at the 1000 μg/mL dose and a 48-h incubation time.

3.2. Donors' responses to controls

PBMC were harvested from healthy blood donors. Six donors were tested with the Colostrum Control, the positive IL-2 control, and the negative solvent control. In Fig. 2, high variability is clearly seen. Donor 5 showed the highest response by this metric. However, this donor also showed an abnormally high response to the negative solvent control

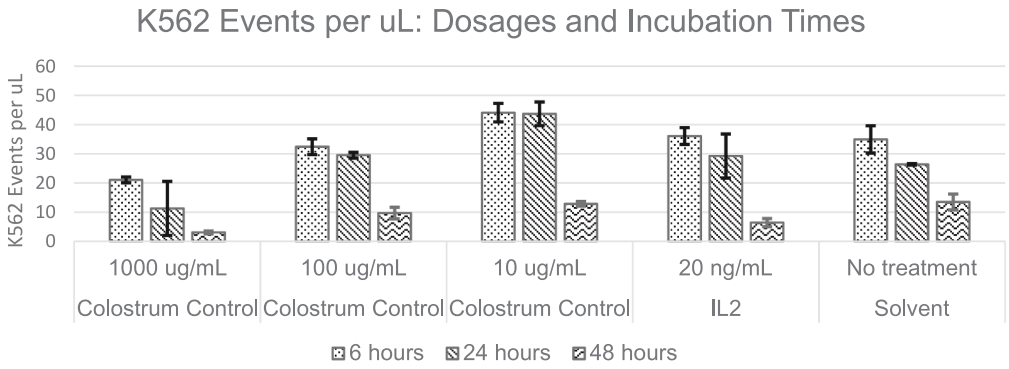

Fig. 1. Dosages and incubation times. Visual representation of the data in Table 1. Shown are absolute counts of K562 target cells. Expressed as the average of 3 measurements ±95% CI.

Table 1
Dosages and incubation times.

Sample		6 Hours		24 Hours		48 Hours	
Test article	Concentration	K562 Events/uL	*p*	K562 Events/uL	*p*	K562 Events/uL	*p*
Colostrum Control	1000 µg/mL	21.1 ± 1.0	0.005	11.3 ± 9.3	0.03	3.1 ± 0.5	0.002
Colostrum Control	100 µg/mL	32.4 ± 2.7	0.4 (NS)	29.5 ± 1.0	0.004	9.7 ± 2.0	0.09 (NS)
Colostrum Control	10 µg/mL	44.1 ± 3.2	0.03	43.7 ± 4.0	0.001	12.9 ± 0.8	0.7 (NS)
IL2	20 ng/mL	36.1 ± 2.9	0.7 (NS)	29.2 ± 7.6	0.5 (NS)	6.4 ± 1.4	0.01
Solvent	No treatment	34.9 ± 4.7	Reference	26.4 ± 0.3	Reference	13.5 ± 2.7	Reference

NK cell response to three dosages of the Colostrum Control (colostrum filtrate) at three different incubation times, alongside the solvent control and the IL-2 control. Shown are absolute counts of K562 target cells, expressed as the average of three measurements ±95% CI, along with *p* values between each test condition and its reference (bottom row). NS = non-significant.

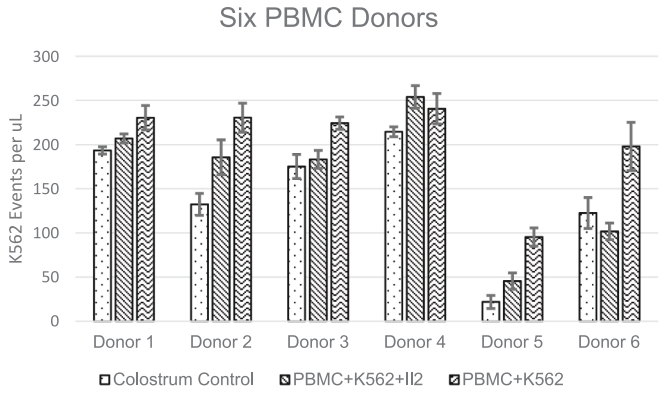

Fig. 2. Responses to six PBMC donors. Six donors' responses to controls. Shown are absolute counts of K562 target cells. Expressed as the average of 3 measurements ±95% CI.

(PBMC + K562) compared to the PBMC from the other donors, measured in absolute K562 counts.

3.3. Lot-to-lot variability, Part I

In order to demonstrate natural variability of the colostrum control ingredient, 11 different batches of the bovine colostrum ingredient were tested after dissolution in PBS pH 7.4. Each lot showed difficulty going into solution, and resulted in a solution with pH 7.0.

The Colostrum Control ingredient showed no response compared to the negative solvent control, as shown in Fig. 3. Seven of the 11 lots tested showed a higher cell count than the negative control, indicating no response for those batches as well (See Fig. 3).

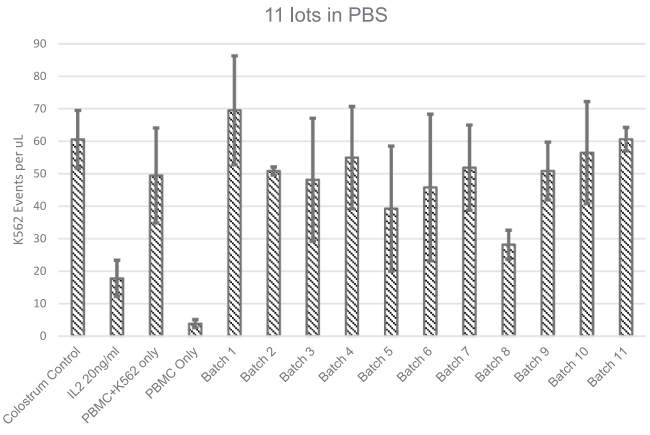

Fig. 3. NK cell response to different batches of bovine colostrum in PBS. Eleven batches of ultra-filtered bovine colostrum solvated in PBS tested alongside controls. Shown are absolute counts of K562 target cells. Expressed as the average of 3 measurements ±95% CI.

3.4. Solubility experiment

In a subsequent round of testing, no issues with solubility were observed in the Colostrum Control sample that was dissolved in PBS pH 7.4 (resulting in a solution with pH 7.0). There were no visible particulates in this sample; all of the compound was in solution or in a very fine suspension presenting with very little cloudiness. Similar solubility was noted in the Colostrum Control in 50 mM citric acid solution, which yielded a solution with pH 2.46.

The Colostrum Control in PBS resulted in 99.7% killing of the K562 cells compared to the solvent control; the sample in citric acid solution resulted in only 61.8% killing (See Fig. 4).

Solubility using different ratios of citric acid solution to PBS was explored, with the best results (determined visually) at a 4 mL:6 mL

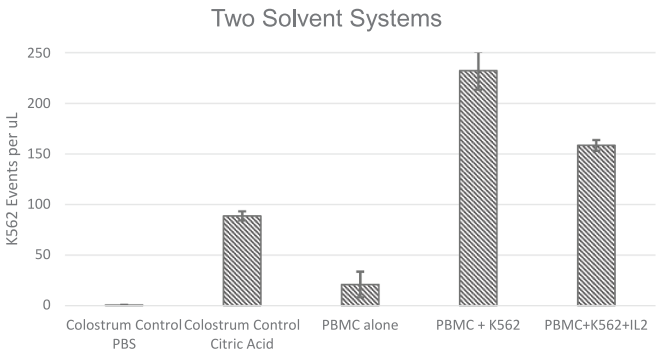

Fig. 4. NK cell response to two different solvent systems. Comparison of two solvent systems side-by-side with two controls. Shown are absolute counts of K562 target cells. Expressed as the average of 3 measurements ±95% CI.

ratio, respectively. This ratio of solvents resulted in the least cloudy solutions, with a pH of 3.2. The results in subsequent rounds of testing were mixed; some samples responded better to this solvent system than to the pure 50 mM citric acid solution, but some did not (data not shown).

3.5. Lot-to-lot variability, Part II

On a later round of testing, the lot-to-lot variability experiment from section 3.3 was repeated with a different solvent system. The 11 lots of bovine colostrum and the Colostrum Control were solubilized in a 50 mM citric acid solution. This solvent system was much more effective than the PBS at getting the compounds into solution.

Results for the different batches showed an average of 38.4% killing (range 23.1% - 51.8%), when all 11 lots were compared to each other, showing natural variability in these lots collected from different dairies at different times of the year. In this round, the Colostrum Control killed 38.1% of the K562 cells, which was a substantially better response than the previous test with PBS as solvent. This response was also in line with the average NK cell response after treatment with these 11 lots (See Fig. 5).

4. Discussion

4.1. Time and dose response

When designing the protocol for these experiments, three incubation times were considered: 6 h, 24 h, and 48 h. In the literature, a 2-h incubation time has been demonstrated (Leh Chang et al., 1993), but this was without stimulation by any substance. In another study using a similar colostrum-based ingredient, incubation times of 18 or 72 h were used in various parts of the experiment (Benson et al., 2012). In order to obtain significantly discernible results, 6 h was selected as the minimum. Due to concerns about the rate of activation from natural products *versus* IL-2, longer incubation times were also tested. The most effective response of the NK cells was achieved with the 48-h incubation time, which was used moving forward with subsequent rounds of testing.

In the previously referenced study (Benson et al., 2012), serial dilutions were performed on the similar colostrum-based ingredient. Taking previously studied methodology into account, three concentrations of the Colostrum Control sample were chosen for testing. The lowest dose was chosen due to its ready solubility. As the concentration of the Colostrum Control in PBS increased, solubility decreased. The aim was to find the lowest dose that would demonstrate an effect on the NK cells that would also have acceptable solubility. In the experiment, the highest dose showed the most significant difference from the solvent control. It stands to reason that this higher dose would be necessary because the immunologically active components of colostrum are at very low relative concentrations. A much higher dose would be needed than if the assay were testing a pure compound, as demonstrated by the response to the IL-2 at only 20 ng/mL. IL-2 is also completely soluble in PBS, as opposed to the Colostrum Control, which is only partially soluble.

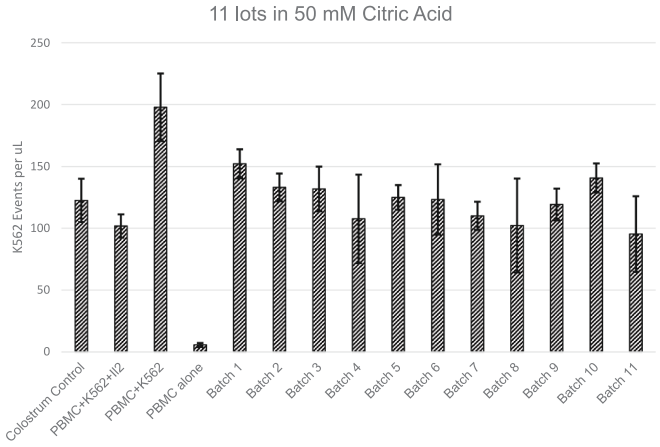

Fig. 5. NK cell response to different batches of bovine colostrum in 50 mM Citric Acid. Same 11 lots of ultra-filtered bovine colostrum tested in 50 mM citric acid solution as solvent instead of PBS. Shown are absolute counts of K562 target cells. Expressed as the average of 3 measurements ±95% CI.

4.2. Donors' response to controls

As PBMCs were harvested from human donors, natural variation in levels of NK cells was expected. (Wullner et al., 2010) In order to determine the effect of this variation on the assay, six healthy donors' samples were tested together in one run. Donor 6 was used in later experiments due to an acceptable response to stimulus, an average response to the solvent control (compared to the other five donors), and availability. Many vials of PBMC from this donor were stored for future experiments. In this case, future experiments might not be able to be directly compared to each other due to a different response from a different, future donor.

4.3. Solubility experiment

The experiment's success appeared to hinge on the solubility of the test compounds. To attempt mitigation of poor solubility of some of the natural products in PBS only, the Colostrum Control was tested side-by-side in both PBS and a 50 mM citric acid solution (Fig. 3). In preparing the samples for this assay, the samples were often not completely soluble in the PBS solvent even with extensive sonication. On occasion this led to undissolved particles showing up in the flow cytometry readouts as extra K562 cells, confounding the results. For this reason, in order to attempt to mitigate this issue, a different solvent was tested alongside the PBS. The 50 mM citric acid solution dissolved some samples better, so the Colostrum Control was tested with both solvents. There was a large difference in NK cell activity. Presumably, this difference is due to either the lower pH in the 50 mM citric acid solvent, or uncharacterized inhibitory activity by the citric acid.

In order to elicit results that were more representative of earlier responses from the Colostrum Control, the hybrid solvent system was developed and tested on colostrum and other samples (data not shown). For the Colostrum Control, better solubility was seen with the new 4:6 citric:PBS solvent ratio than with either PBS or citric acid solution alone.

4.4. Batch-to-batch variability

Due to the poor solubility of the compounds, the active components of the colostrum may not have been dissolved well enough to stimulate

the NK cells. This is likely the reason why activity was low, or in some cases, non-existent. Another theory is that undissolved particulates could have been present that artificially increased the K562 cell count.

Due to the poor response of the colostrum ingredient in this round of testing, the solubility experiment was performed and based on those results, the same samples were tested in citric acid solution, yielding quite different results. The average response of the 11 batches was very close to the response to the Colostrum Control, indicating that solubility was not only better in the new solvent system but also more consistent between samples. The variability that was observed within the 11 samples was expected, as the different batches were all harvested from different dairies and at different times of the year, most likely affecting the concentrations of the actives in the material.

4.5. Limitations and assumptions

In considering the results we obtained from each experiment, it is important to keep in mind the limitations of the assay. One of the assumptions made is that similar results will be obtained from run to run, which is not always the case as demonstrated by the varied responses to the Colostrum Control, the purpose of which was to compare data between runs. Variability in any natural compounds tested, including the Colostrum Control, are to be expected, not only due to the complex nature of the sample but also due to different natural concentrations and activity of transfer factors, Igs, and other immunologically active substances present in the compounds. These variations could affect not only the NK cell response to stimulus, but also the solubility of the natural products. This could mean that future experiments using a different batch of the Colostrum Control ingredient could not necessarily be compared directly with past experiments.

Another limitation is the natural variability in the unstimulated and stimulated NK cell response of the different PBMC donors. It would be possible to find a new donor that has a similar response to the current donor by repeating the donor variability experiment with new batches of PBMC and looking for a similar response. Doing so could decrease variability between runs. There is the risk, however, that a donor with matching capabilities could not be found.

Solubility issues could be addressed by testing different solvent systems than those tested here. Potential solvent systems for the compounds tested are limited to aqueous solutions, as to be compatible with the rest of the reagents in the assay. Acidifying the solution with citric acid seemed to help in many cases. In the future other organic acids could be used to attempt to solubilize the compounds without changing the pH of the samples enough to inhibit NK cell activity. If this resulted in an increased activity of NK cells compared to the citric acid solvent, it might shed light on the reason the activity was so much lower with the citric acid solvent than with the PBS solvent in the solubility experiment.

Overall, the success of the experiment appeared to hinge on solubility. This is a serious limitation on the accuracy of the method. Differences in solubility were an issue from experiment to experiment even when preparing the Colostrum Control, which was prepared from the same powder sample every time. As demonstrated by the vastly different results obtained from the 11 samples in PBS *versus* citric acid solvent, the measured NK cell response relied heavily on the ability to get the compounds into solution.

This method was developed to create a screening process for biological compounds before introduction to living systems. The literature cited gives evidence that these compounds will survive digestion. We will be validating these reports in future research.

5. Conclusion

A new method using flow cytometry to determine the immunological activity of natural products was developed. A few parameters such as different incubation times, doses, PBMC sources, and solvent systems were tested. Many limitations exist for this novel method, including PBMC variability, natural product variability, and especially the solubility of test compounds. Optimizing these parameters and addressing these limitations requires further research, after which activity of this compound can be tested in an *in vivo* setting.

Declaration of Competing Interest

Authors and principle investigators are employed by 4Life Research, which sells the test articles commercially. 4Life Research funded all portions of this study.

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All testing was performed at ReachBio Research Laboratories.

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