

## Short Communication

# Trastuzumab Made in Plants Using *vivoXPRESS*® Platform Technology

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**Abstract**

PlantForm Corporation's *vivoXPRESS*® platform technology uses *Nicotiana benthamiana* plants to produce therapeutic protein-based pharmaceutical drugs. Processes to control post-translational glycomodification of proteins by these plants have also been developed to produce therapeutic protein drugs with potential for enhanced medicinal effects, as antibodies produced without  $\alpha$ 1,6-fucosylation have been demonstrated to have improved antibody-dependent cell-mediated cytotoxicity (ADCC) activity. As shown here, the *vivoXPRESS*® platform is capable of producing trastuzumab antibodies with equivalent purity and functionality compared to the innovator drug Herceptin®.

**ABBREVIATIONS**

ACCN: Acetonitrile; ADCC: Antibody-Dependent Cell-Mediated Cytotoxicity; CFSE: Carboxyfluorescein; Cm: Centimeter; Da: Dalton; DDA: Data-Dependent Acquisition; EIC: Extracted Ion Chromatogram; FBS: Fetal Bovine Serum; FACS: Fluorescence-Activated Cell Sorting; HC: Heavy Chain; HER2: Human Epidermal Growth Factor 2; HPLC: High-Performance Liquid Chromatography; Hrs: Hours; ID: Internal Diameter; IgG: Immunoglobulin G; IL2: Interleukin 2; IVIG: Intravenous Immunoglobulin; KDa: Kilodalton; LC: Light Chain; LC-ESI-MS: Liquid Chromatography-Electrospray Ionization-Mass Spectrometry; Min: Minute; Ml: Milliliter; Mm: Millimeter; Mm: Millimolar; Mu: Milli Unit; PAGE: Polyacrylamide Gel Electrophoresis; PBMC: Peripheral Blood Mononuclear Cell; PBS: Phosphate-Buffered Saline; PFC: Plantform Corporation; PI: Propidium Iodide; QTOF: Quadrupole Time-Of-Flight; SDS: Sodium Dodecyl Sulfate; SEC: Size-Exclusion Chromatography; SEM: Standard Error Of The Mean; W/V: Weight Per Volume;  $\mu$ G: Microgram;  $\mu$ L: Microliter

**INTRODUCTION**

The scientific area concerned with plant-based production of therapeutic proteins has matured significantly since Hiatt et al., first demonstrated expression of an IgG antibody in tobacco plants [1]. This year, the seventh biennial conference on Plant-Based Vaccines, Antibodies and Biologics will be held in Albufeira, Portugal ([http://www.meetingsmanagement.co.uk/index.php?option=com\\_content&view=article&id=366&Itemid=721](http://www.meetingsmanagement.co.uk/index.php?option=com_content&view=article&id=366&Itemid=721)). It is anticipated that this conference will have even greater attendance than the 2015 conference in Lausanne, Switzerland,

which attracted participants from academia and more than 20 companies engaged in development of therapeutic protein-based drug products from plants. From our perspective, continued improvements in plant biotechnology now offer distinct advantages over microbial and mammalian cell culture methods for production of therapeutic proteins. It is inevitable that plant-based production will soon provide global pharmaceutical markets with a multitude of diverse innovator and biosimilar biologic drugs.

**MATERIALS AND METHODS****Growth of plants, SDS-PAGE, SKBR3 cell growth-arrest assay**

As published [2].

**Expression and purification of trastuzumab**

According to proprietary methodology (PlantForm *vivoXPRESS*® technology; see [www.plantformcorp.com](http://www.plantformcorp.com)).

**Size-exclusion chromatography (SEC)**

Samples were run on a TSKgel® G3000SW<sub>XL</sub>, 5  $\mu$ m particle size, 7.8 mm ID x 30 cm column (Tosoh Bioscience) using an Agilent 1100 Series HPLC (Agilent Technologies). Mobile phase: 50mM sodium phosphate buffer pH 6.8, 0.05% sodium azide (w/v), 150mM sodium chloride. Injection volume: <10  $\mu$ L (5 - 10  $\mu$ g). Flow rate: 0.3mL/min. Herceptin® was purchased from Roche.

**Antibody-dependent cell-mediated cytotoxicity (ADCC) assay**

SKBR3 cells were seeded in flat bottom 96-well plates one

day prior to antibody treatment at  $2 \times 10^4$  cells per well in 200  $\mu\text{L}$  of culture medium. PBMCs were purified from fresh buffy coats prepared from three healthy donors using Ficoll gradient centrifugation, washed three times with PBS and resuspended in RPMI medium containing 10% heat inactivated FBS and IL2. Cells were counted, and viability was determined by the Trypan Blue exclusion. SKBR3 cells were trypsinized to verify cell count in order to determine the number of viable PBMCs required for 25:1 effector: target (E:T) ratio. Target cells were labeled with CFSE immediately prior to treatment. Antibodies were added to cells at concentrations ranging from 10 ng to 10  $\mu\text{g}/\text{mL}$  and incubated for 15 min. PBMCs were then added to wells at 25:1 (E:T ratio), and plates briefly spun down. Plates were incubated for four hours. Following treatment, plates were transferred to 4°C. Cells were harvested and added to 400  $\mu\text{L}$  PBS containing propidium iodide (PI). FACS analysis measured the proportion of PI+ green cells (targets) as a fraction of total target cells. Average ADCC activities from three replicate counts for each data point are given along with standard error bars (SEM).

### Glyco analysis by LC-ESI-MS

Proteins were S-alkylated with iodoacetamide *in situ* (PAGE gel) and digested with trypsin (Promega).  $\beta$ -galactosidase (*Aspergillus oryzae*, specific for  $\beta$ ,1-4 linkages) digestion was also performed (16 hrs at 37°C of 15  $\mu\text{g}$  tryptic digested protein with 13.8 mU of enzyme) to elucidate percentage of galactosylation. Digested samples were loaded on a BioBasic C18 column (BioBasic-18, 150 x 0.32 mm, 5  $\mu\text{m}$ , Thermo Scientific) using 65 mM ammonium formate buffer as the aqueous solvent. A gradient from 5% B (B: 100% ACCN) to 32% B in 35 min was applied, followed by a 15 min gradient from 32% B to 75% B that facilitates elution of large peptides, at a flow rate of 6  $\mu\text{L}/\text{min}$ . Detection was performed with QTOF MS (Bruker maXis 4G) equipped with the standard ESI source in positive ion, DDA mode (= switching to MSMS mode for eluting peaks). MS-scans were recorded (range: 150-2200 Da) and the 6 highest peaks were selected for fragmentation. Instrument calibration was performed using ESI calibration mixture (Agilent). Manual glycopeptide searches were made using Data Analysis 4.0 (Bruker). For the quantification of the different glycoforms the peak areas of EIC (Extracted Ion Chromatograms) of the first 4 isotopic peaks were summed.

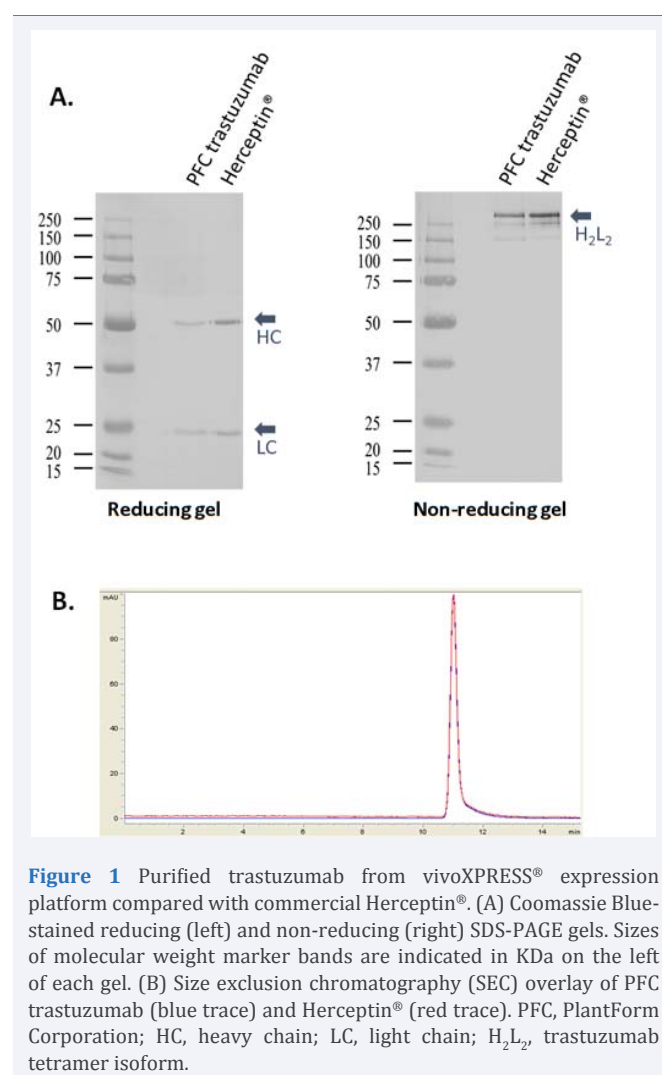
## RESULTS AND DISCUSSION

PlantForm Corporation uses a transient expression system involving its own *Nicotiana benthamiana* plant line engineered for knock down of plant-specific  $\alpha$ 1,3-fucosylation and  $\beta$ 1,2-xylosylation to produce vaccine, antibody and biologic drugs based on technology originally developed at the University of Guelph [3,4]. The company has continually improved this technology since its incorporation, achieving increases upwards of 10,000-fold for recombinant protein expression as well as controlled additions of post-translational glycomodifications to target protein products.

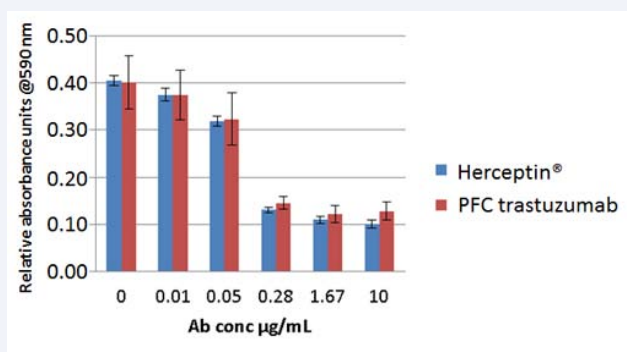
Trastuzumab produced in plants using this technology has equivalent purity with commercial Herceptin® as seen by SDS-PAGE and SEC analyses (Figure 1). Multiple lots of plant-produced trastuzumab have been shown to have equivalent

activity with commercial Herceptin® for both HER2 binding and HER2-positive cell growth arrests, as can be seen in Figure (2) as well as in a previous publication [2].

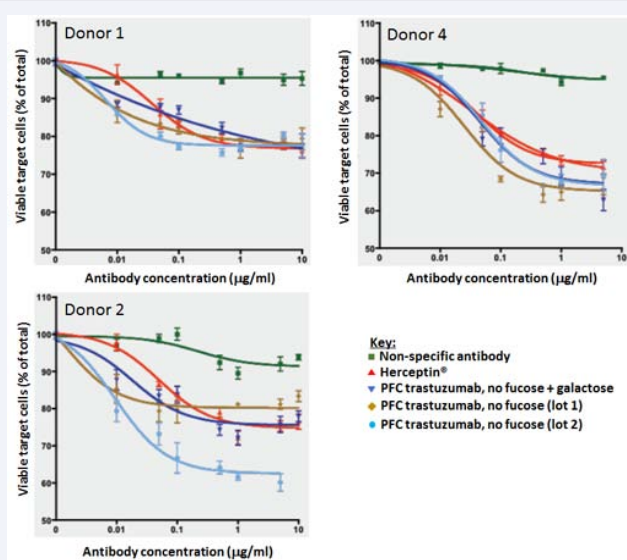
Three plant-produced antibodies, a non-specific antibody (negative control) and commercial Herceptin® were analyzed for antibody-dependent cell-mediated cytotoxicity (ADCC) activity using SKBR3 target cells and effector cells from healthy individual donors (Figure 3). In these experiments, the negative control antibody consistently failed to induce ADCC, whereas commercial Herceptin® demonstrated predicted ADCC activity for all effector cell samples tested. Plant trastuzumab, produced either without fucose (two lots) or without fucose but with galactose demonstrated equivalent ADCC activities compared to commercial Herceptin® except for one plant lot without fucose (lot 2) which was better than all other antibodies with PBMCs from Donor 2. More ADCC assays would need to be performed to determine whether this single instance is of significance. No plant specific  $\alpha$ 1,3-fucose or  $\beta$ 1,2-xylose glycans were detected on any of these plant trastuzumab lots. See Table (1) for percentages of N-linked glycan species on samples of trastuzumab used in this ADCC assay. Table 1 also gives percentages for total human serum IgG glycans (from IVIG) and for total human Fc glycans (from IgG).



**Figure 1** Purified trastuzumab from vivoXPRESS® expression platform compared with commercial Herceptin®. (A) Coomassie Blue-stained reducing (left) and non-reducing (right) SDS-PAGE gels. Sizes of molecular weight marker bands are indicated in kDa on the left of each gel. (B) Size exclusion chromatography (SEC) overlay of PFC trastuzumab (blue trace) and Herceptin® (red trace). PFC, PlantForm Corporation; HC, heavy chain; LC, light chain; H<sub>2</sub>L<sub>2</sub>, trastuzumab tetramer isoform.



**Figure 2** PFC trastuzumab shows similar inhibitory activity to commercial Herceptin® on in vitro growth of HER2 positive breast cancer cell line SKBR3. Experiment performed by an independent outside provider. Histogram bars indicate average of three measurements +/- standard errors (SEM).



**Figure 3** PFC trastuzumab shows similar antibody-dependent cell-mediated cytotoxicity (ADCC) to commercial Herceptin®. Data charts for antibody treatments and effector cells from three healthy donors are given (with standard errors for each data point; SEM). Refer to color key at top of figure. The negative control involved a non-specific antibody. Percentages of glycan species for trastuzumab antibodies are given in (Table 1).

**Table 1:** Abundance of N-linked glycan species on trastuzumab samples and human serum IgG antibodies

Antibody		GnGn <sup>1</sup>	GnGnF	AA	AGnF/ GnAF	AAF	Man9	Minor glycans (sum %)
Source	Type	(%)	(%)	(%)	(%)	(%)	(%)	
PFC, no fucose (lot 1)	Trastuzumab <sup>2</sup>	70.8						29.2
PFC, no fucose (lot 2)	Trastuzumab <sup>2</sup>	77.5						22.5
PFC, no fucose plus galactose	Trastuzumab <sup>2</sup>	36.6		19.3			6.3	37.8
Herceptin® <sup>3</sup>	Trastuzumab <sup>2</sup>	3.8	33.5		30.4	3.4		28.9
Human serum	Total IgG (IVIG) <sup>4</sup>	0.2	13.5	2.1	35.1	22.0		27.1
Human serum	Total IgG1 (Fc) <sup>4</sup>	0.1	10.1	1.5	33.1	24.0		31.2

<sup>1</sup>For glycan structures and nomenclature visit:

[http://www.proglycan.com/sites/default/public/pdf/IgG\\_Table\\_Rosetta.pdf](http://www.proglycan.com/sites/default/public/pdf/IgG_Table_Rosetta.pdf)

<sup>2</sup>Determined by LC-ESI-MS analysis (this work)

<sup>3</sup>Purchased from Roche.

<sup>4</sup>Human serum glycan species abundances taken from

[http://www.proglycan.com/sites/default/public/pdf/IgG\\_Table\\_Rosetta.pdf](http://www.proglycan.com/sites/default/public/pdf/IgG_Table_Rosetta.pdf)

**Abbreviations:** IgG: Immunoglobulin G; IVIG: Intravenous Immunoglobulin; LC-ESI-MS: Liquid Chromatography-Electrospray Ionization-Mass Spectrometry; PFC: PlantForm Corporation

## CONCLUSION

Trastuzumab produced in *N. benthamiana* plants using PlantForm's *vivoXPRESS*<sup>®</sup> platform technology, without fucose and with or without galactose, has equivalent ADCC activity to commercial Herceptin<sup>®</sup>. It is possible that trastuzumab produced without fucose could have better ADCC activity than commercial Herceptin<sup>®</sup>, as suggested in published literature showing that  $\alpha$ 1,6-fucosylation has a negative effect on this immunological function [5]. Post-translational modification of glycoprotein drugs is complicated, as exemplified by commercial Herceptin<sup>®</sup> (Table 1). This highly effective anti-cancer drug possesses different glycomodifications compared to those on human serum IgGs, most notably having 20% more GnGnF glycans and 20% less AAF glycans. This indicates a degree of flexibility for the glycans of protein-based drugs, which should allow PlantForm to develop processes using its *vivoXPRESS*<sup>®</sup> platform to produce trastuzumab products with either biosimilar or biobetter activities than the innovator version of this drug.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The author is one of PlantForm Corporation's original founders and has a financial interest in the company.

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