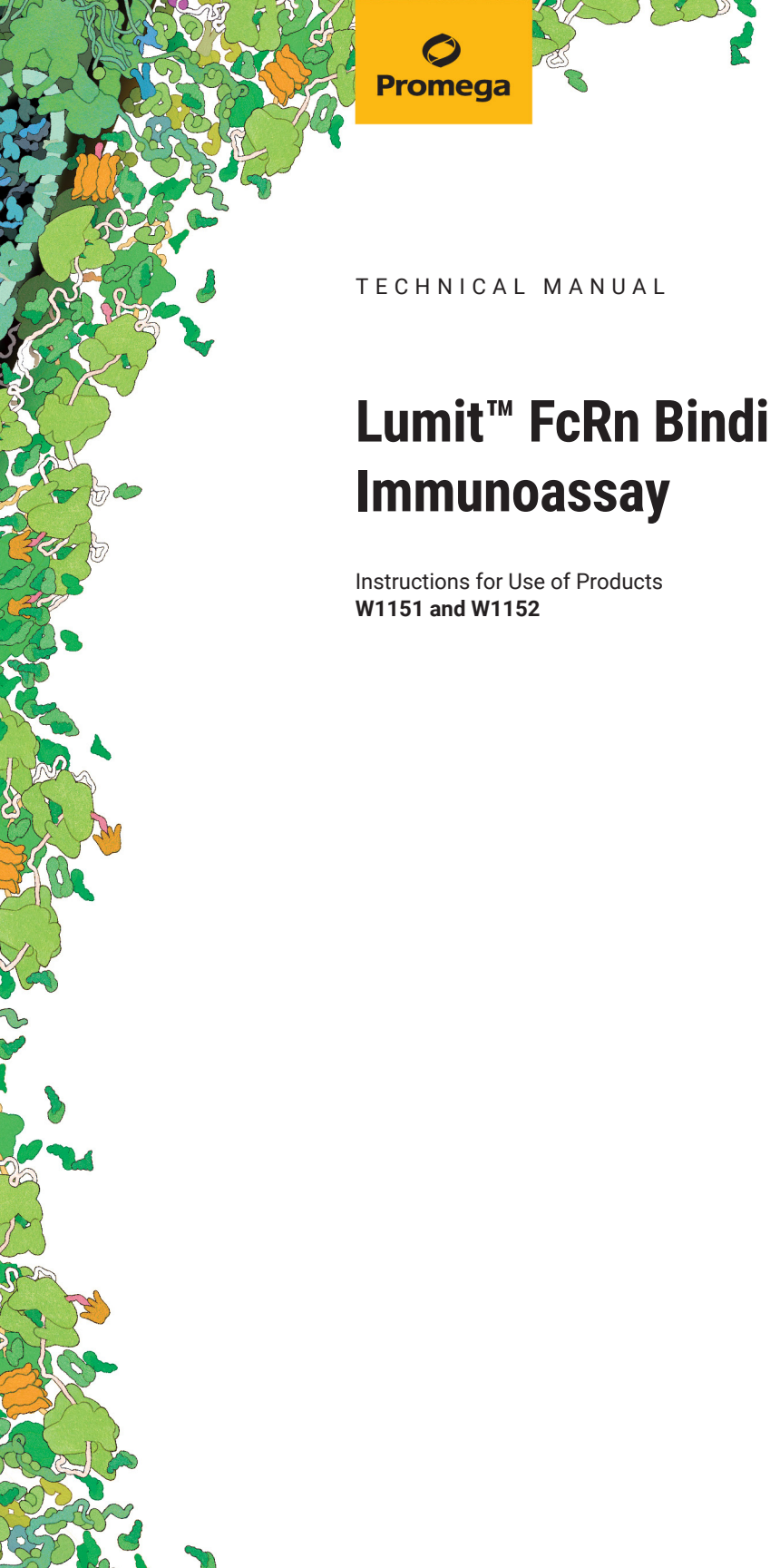

TECHNICAL MANUAL

Lumit™ FcRn Binding Immunoassay

Instructions for Use of Products
W1151 and W1152

Revised 7/22
TM611

Lumit™ FcRn Binding Immunoassay

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The neonatal Fc receptor (FcRn) is a major histocompatibility complex (MHC) class I-like heterodimeric protein comprised of a light chain β_2-microglobulin (β_2m) and a transmembrane heavy chain (α-FcRn). FcRn is expressed in the endosomal compartments of a variety of cell types, including vascular endothelium and antigen-presenting cells (APCs). FcRn binds to the Fc region of immunoglobulin G (IgG) antibodies at acidic pH within endosomes. In utero, FcRn acts to transfer maternal IgG to the developing fetus. In adults, it is involved in recycling of IgG and albumin. Recycling by FcRn is the primary reason for the long half-life (several weeks) of IgG and albumin in serum. Furthermore, a critical factor for the success of therapeutic antibodies is their extended half-life, which contributes to efficacy and improved dosing schedule. Therefore, the FcRn-IgG interaction is a key parameter to optimize and track throughout the antibody drug development process.

The Lumit™ FcRn Binding Immunoassay[a,b] is a novel homogeneous (no-wash) competition assay, based on NanoBiT® technology (1) to measure the interaction between human FcRn and Fc proteins, including antibodies (Figure 1). NanoLuc® Binary Technology (NanoBiT) is a structural complementation reporter designed for biomolecular interaction studies. The NanoBiT® system is composed of two subunits, the 18kDa Large BiT (LgBiT) and the 11 amino acid peptide Small BiT (SmBiT) that have been optimized for stability and minimal self-association.

In the Lumit™ FcRn Binding Immunoassay, a human IgG1 labeled with LgBiT (Tracer-LgBiT) is used as the tracer. A C-terminal biotinylated human FcRn bound to streptavidin-SmBiT (hFcRn-SmBiT) is used as the target. In the absence of an antibody analyte, Tracer-LgBiT binds to the hFcRn-SmBiT target, resulting in maximum luminescence signal. In samples containing analyte, nonlabeled IgG will compete with Tracer-LgBiT for binding to the FcRn target, resulting in a concentration-dependent decrease in the luminescent signal.

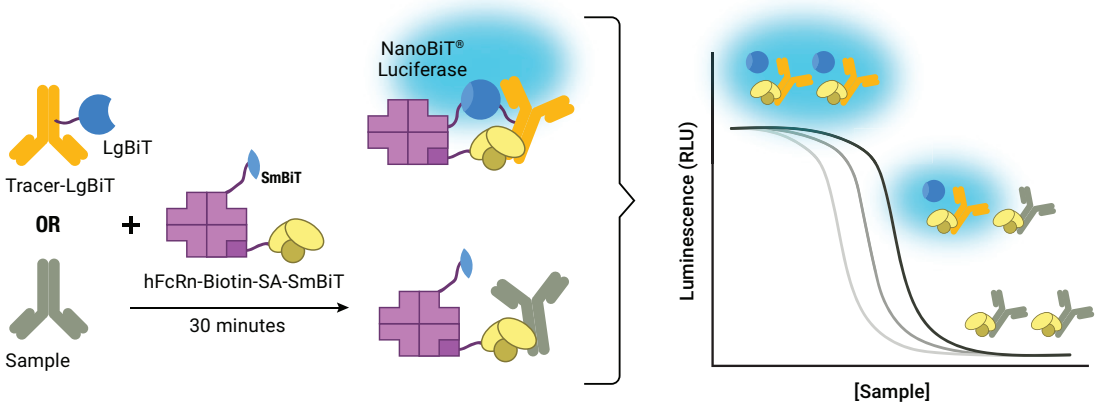

Figure 1. Schematic of the Lumit™ FcRn Binding Immunoassay.

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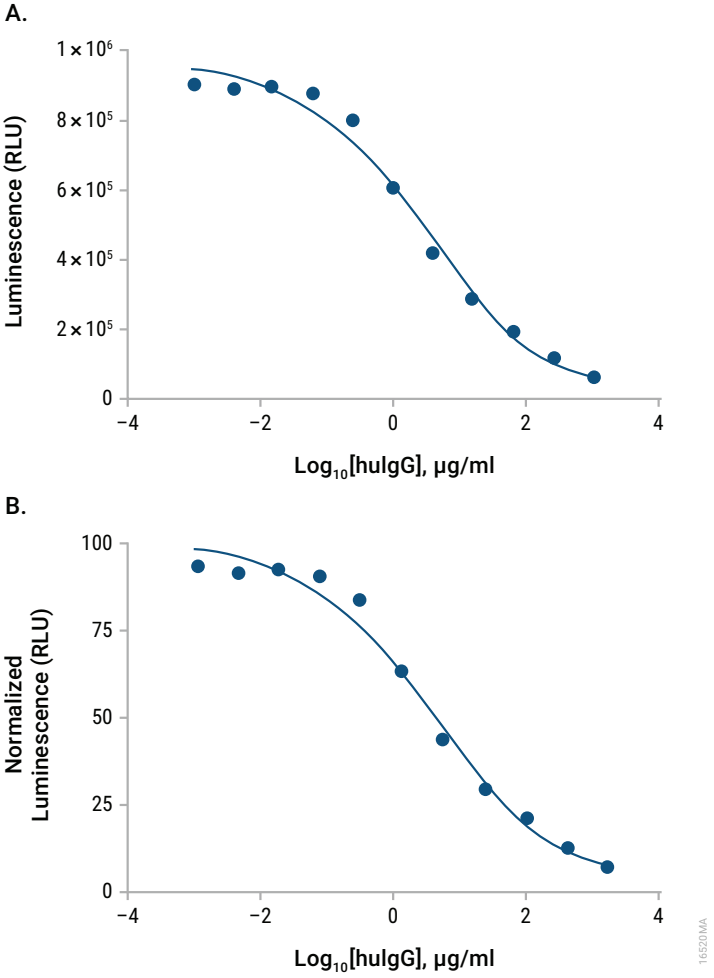

Figure 2. The Lumit™ FcRn Binding Immunoassay detects IgG-FcRn interaction. This is a representative standard curve of control antibody plotted with raw RLU (**Panel A**) and as normalized data (**Panel B**). Normalized luminescence is calculated by assigning 100% to the maximum bioluminescent signal obtained in the absence of an analyte and then calculating percentage drop in signal in the presence of an analyte. Data were fitted to a four-parameter logistic regression equation to calculate IC_{50}. HuIgG is human polyclonal IgG. **Note:** 1.0µg/ml of huIgG = 6.7nM.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Lumit™ FcRn Binding Immunoassay	**1 each**	**W1151**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 100 assays using a 96-well plate.
Includes:

- 120µl Control Ab (red cap)
- 60µl Tracer-LgBiT (green cap)
- 12µl hFcRn-Biotin (dark blue cap)
- 12µl Streptavidin-SmBiT (light blue cap)
- 75µl Lumit™ Detection Substrate A (brown tube)
- 250µl pH Adjustment Buffer (orange cap)
- 25ml FcRn Assay Buffer (bottle)

PRODUCT	SIZE	CAT.#
Lumit™ FcRn Binding Immunoassay 10X	**1 each**	**W1152**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 1,000 assays using 96-well plates.
Includes:

- 10 × 120µl Control Ab (red cap)
- 10 × 60µl Tracer-LgBiT (green cap)
- 10 × 12µl hFcRn-Biotin (dark blue cap)
- 10 × 12µl Streptavidin-SmBiT (light blue cap)
- 10 × 75µl Lumit™ Detection Substrate A (brown tube)
- 10 × 250µl pH Adjustment Buffer (orange cap)
- 10 × 25ml FcRn Assay Buffer (bottle)

Storage Conditions: Store kit at –30°C to –10°C.

Note: Centrifuge tubes briefly to collect reagents at the bottom of the tube before use.

3. Before You Begin

Please read through the entire protocol to become familiar with the components and the assay procedure before beginning.

The Lumit™ FcRn Binding Immunoassay is intended for use with user-provided antibodies and Fc fusion proteins. A polyclonal antibody is provided in the kit to use as a positive control antibody. If a true standard is required, the NISTmAb reference material is available from the National Institute of Standards and Technology (Cat.# RM8671). Affinity between Human IgG and FcRn is relatively weak (approximately 100–300nM). In order to get a full sigmoidal dose-response curve, you may need to adjust the dilution series suggested in Table 1.

FcRn binds antibodies at pH 6.0. Therefore, it is important that your sample antibody be at pH 6.0. For samples in PBS, add 1/10th the volume of pH Adjustment Buffer to bring the sample to pH 6.0. For samples in other buffers, we recommend buffer-exchanging the sample with PBS (pH 7.2), then adding 1/10th the volume of pH Adjustment Buffer to bring the sample to pH 6.0.

The Lumit™ FcRn Binding Immunoassay produces a bioluminescent signal and requires a sensitive luminescence plate reader for signal detection. Performance data included in this Technical Manual were generated using the GloMax® Discover System luminometer (Section 7, Related Products). An integration time of 0.5 seconds/well was used for all readings. The assay is compatible with most other plate-reading luminometers; however, relative luminescence unit (RLU) readings may vary due to the sensitivity and settings of each instrument. The use of different instruments should not affect the measured relative potency of test samples.

4. Lumit™ FcRn Binding Immunoassay Protocol

Materials to Be Supplied by the User

- white, multiwell assay plates, preferably polypropylene or nonbinding (e.g., Corning® Cat.# 3605)
- multichannel pipette or automated pipetting station
- luminometer capable of reading multiwell plates (e.g., GloMax® Discover System, Cat.# GM3000)
- reagent reservoirs (e.g., Thermo Fisher Scientific Cat.# 8093-11)
- plate sealer (e.g., Microtiter™ Plate Sealer, Thermo Fisher Scientific Cat.# 3501)
- plate shaker (e.g., IKA MTS 2/4 digital microtiter shaker, ID.# 0003208001)
- **optional:** NISTmAb reference material (NIST Cat.# RM8671)

Note: After thawing, keep all kit components on ice.

4. Lumit™ FcRn Binding Immunoassay Protocol (continued)

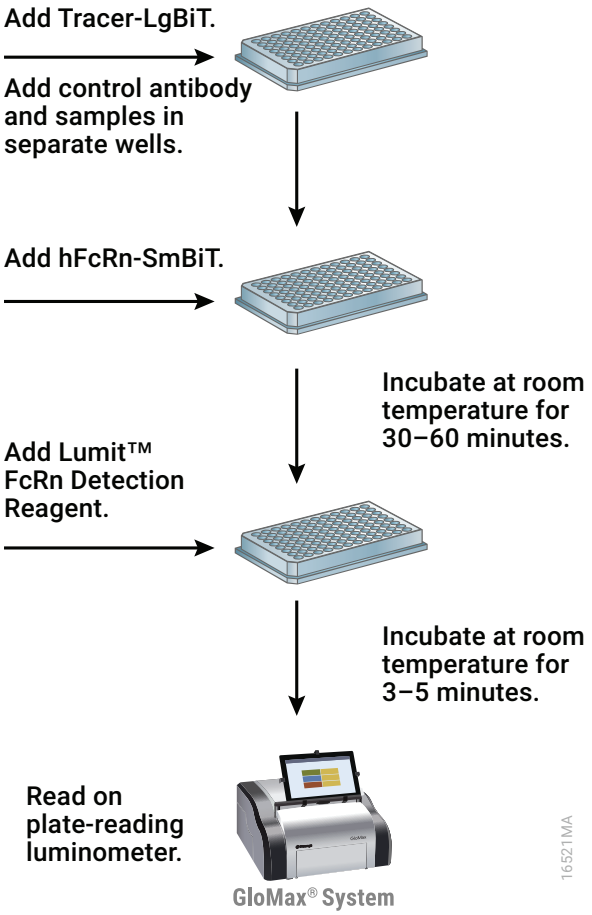

Figure 3. Lumit™ FcRn Binding Immunoassay schematic.

4.A. Preparing the Control Antibody

A 12-point standard curve is recommended for each plate. Use Table 1 as a guide to prepare Control Antibody dilutions. The Control Antibody is at pH 6.0; no additional buffer exchange is necessary. There will be enough antibody for two replicates. Scale as necessary for the number of plates needed.

Table 1. Preparing Antibody Control.

Tube/Well #	FcRn Assay Buffer (µl)	Volume and Tube # of Control Antibody (µl)	Initial IgG Concentration (µg/ml)	Final IgG Concentration (µg/ml)
1	0	100 of Red tube	4,000	1,000
2	75	25 of #1	1,000	250
3	75	25 of #2	250	62.5
4	75	25 of #3	62.5	15.6
5	75	25 of #4	15.6	3.9
6	75	25 of #5	3.9	1.0
7	75	25 of #6	1.0	0.25
8	75	25 of #7	0.24	0.06
9	75	25 of #8	0.061	0.015
10	75	25 of #9	0.015	0.004
11	75	25 of #10	0.004	0.001
12	75	0	0	0

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4.B. Preparing the Sample

Notes:

- When preparing samples, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we find that a sample range of 0–1.0mg/ml with serial fourfold dilutions achieves a full dose-response curve as a 12-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.
- Each kit has sufficient reagents to run a full 96-well plate assay. However, the assay can be scaled down to a 96-well half-area plate, or to a 384-well plate while maintaining the same ratio of reagents.
- FcRn-IgG binding is pH dependent and improper pH adjustment will lead to artifacts.

1. Adjust the sample to pH 6.0. For samples in PBS, add 1/10th the volume of pH Adjustment Buffer to bring the sample to pH 6.0. For samples in other buffers, we recommend buffer-exchanging the sample with PBS (pH 7.2) and then adding 1/10th the volume of pH Adjustment Buffer to bring the sample to pH 6.0.
2. Prepare a serial dilution of the sample in FcRn Assay Buffer. Table 1 provides general guidance on generating a sample dilution series.

4.C. Preparing Reagents

Preparing Tracer-LgBiT

1. Add 3ml of FcRn Assay Buffer to a reagent reservoir. Then add 45µl of Tracer-LgBiT (green cap).
2. Mix the reservoir contents by pipetting.

Preparing hFcRn-SmBiT

3. Add 6ml of FcRn Assay Buffer to a second reservoir. Then add 7.5µl of hFcRn-Biotin (dark blue cap) and 7.5µl of Streptavidin-SmBiT (light blue cap) to the FcRn Assay Buffer in this reservoir.
4. Mix the reservoir contents by pipetting.

4.D. Lumit™ FcRn Binding Immunoassay

1. Pipet 25µl of Tracer-LgBiT solution into the wells of a white 96-well plate.
2. Add 25µl of Control Antibody or sample to each well.
3. Add 50µl of hFcRn-SmBiT solution to each well.
4. Cover the plate with a plate seal and mix gently on a plate shaker (300–400rpm) for 30–60 minutes at room temperature.
5. Add 3ml of FcRn Assay Buffer to a reservoir, then add 60µl of Lumit™ FcRn Detection Substrate A, to create Lumit™ FcRn Detection Reagent. Mix reservoir contents by pipetting.
6. Add 25µl of Lumit™ FcRn Detection Reagent from the reservoir to each plate well.
7. Incubate the plate at room temperature for 3–5 minutes.
8. Read the plate on a luminometer.

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
No binding curve is obtained	FcRn binds antibodies at pH 6.0. Adjust pH of antibody with pH Adjustment Buffer as described in Section 4.B. Significant reductions in binding and changes in IC_{50} can occur at pH >6.0.
	Buffer components in the sample antibody are inhibiting binding to FcRn. Buffer-exchange the sample with PBS (pH 7.2) and then add 1/10th the volume of pH Adjustment Buffer to bring the sample to pH 6.0.
High RLU for entire plate	The Lumit™ FcRn Binding Immunoassay is a competition assay. The order of reagent additions is very important. Follow the protocol for reagent addition order: tracer-LgBiT, sample, then hFcRn-SmBiT.
Inconsistent data points	Pipetting errors.

6. Reference

1. Dixon, A.S. *et al.* (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11(2),** 400–8.

7. Related Products

Lumit™ Immunoassays

Product	Size	Cat.#
Lumit™ Immunoassay Labeling Kit	1 each	VB2500
Lumit™ Immunoassay Cellular System – Starter Kit	200 assays	W1220
Lumit™ Immunoassay Cellular System – Set 1	100 assays	W1201
	1,000 assays	W1202
	10,000 assays	W1203
Lumit™ Immunoassay Cellular System – Set 2	100 assays	W1331
	1,000 assays	W1332
	10,000 assays	W1333
Lumit™ Immunoassay Lysis and Detection Kit	100 assays	W1231
	1,000 assays	W1232
	10,000 assays	W1233

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Lumit™ Immunoassay Detection Reagents

Product	Size	Cat.#
Lumit™ Immunoassay Detection Reagent A	500 assays	VB2010
Lumit™ Immunoassay Detection Reagent A 10X	5,000 assays	VB2020
Lumit™ Immunoassay Detection Reagent A 100X	50,000 assays	VB2030
Lumit™ Immunoassay Detection Reagent B	100 assays	VB4050
Lumit™ Immunoassay Detection Reagent B 10X	1,000 assays	VB4060

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Lumit™ Immunoassay Reagents

Product	Size	Cat.#
Lumit™ Anti-Mouse Ab-LgBiT	30µl	W1021
	300µl	W1022
Lumit™ Anti-Rabbit Ab-SmBiT	30µl	W1031
	300µl	W1032
Lumit™ Anti-Rabbit Ab-LgBiT	30µl	W1041
	300µl	W1042
Lumit™ Anti-Mouse Ab-SmBiT	30µl	W1051
	300µl	W1052
Lumit™ Anti-Goat Ab-LgBiT	30µl	W1061
	300µl	W1062
Lumit™ Anti-Goat Ab-SmBiT	30µl	W1071
	300µl	W1072

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
FcγRIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIa-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991

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Additional kit formats are available.

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000

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8. Summary of Changes

The following changes were made to the 7/22 revision of this document:

1. In Section 4, Materials to Be Supplied by the User, “polypropylene” was added to the description of multiwell plates.
2. Font was updated.

[a]U.S. Pat. Nos. 9,797,889; 9,797,890; 10,107,800; and other patents and patents pending.

[b]U.S. Pat. No. 8,809,529, European Pat. No. 2635582, and other patents and patents pending.