Pleximmune™

Read the entire Package Insert prior to ordering the test. Conformance with the test procedure is necessary to ensure accurate results.

Name and Intended Use:
Pleximmune™ is intended to be performed at a single laboratory to measure the CD154 expression on T-cytotoxic Memory cells (TcM) in patient's peripheral blood lymphocytes (PBL) isolated from heparinized whole blood (anticoagulant – sodium heparin). Pleximmune™ is a qualitative prognostic test intended to be used in patients less than 21 years old with liver or small bowel transplantation. The Pleximmune™ test is an aid in the evaluation of the risk of acute cellular rejection (ACR) and must be used in conjunction with biopsy, standard clinical assessment and other laboratory information.

Pleximmune™ is intended for use at the following time periods:

- Pre-transplantation period: For blood samples collected before transplantation, the test predicts the risk of transplant rejection within 60 days after transplantation.
- Early and late post-transplantation period: For blood samples collected within 60 days (early) after transplantation and for blood samples collected at 200 or more days (late) after transplantation, the test predicts the risk of transplant rejection within 60 days after sampling.

Humanitarian Device. Authorized by Federal Law for use as an aid in the evaluation of the immunological risk for Acute Cellular Rejection (ACR) in children with liver or small bowel transplantation. The effectiveness of this device for this use has not been demonstrated.

Summary and Explanation of the Assay

- The **Pleximmune™** test system uses *in vitro* cell culture to elicit the inflammatory immune response of the recipient to the donor. This inflammatory response then can be measured as a rejection-risk signal in individual children with liver or small bowel transplantation. The test is based on lymphocyte co-culture studies in pediatric liver recipients, which show that rejection is associated with enhanced donor-specific alloreactivity (1-3). This inflammatory response to donor is measured by T-cytotoxic memory cells (TcM) from the recipient, which express the inflammatory marker, CD154 (CD154+TcM) (4-6).
- To determine whether CD154+TcM induced by donor are "increased" as is seen in rejection, or "decreased" as is seen in non-rejection, test results must be expressed differently. This is so because the absolute number of CD154+TcM induced by stimulation with donor cells can vary widely between individuals, so that the number associated with rejection in one person may be associated with non-rejection in the other.
- To characterize rejection-risk in the individual recipient as increased or decreased, the recipient's inflammatory response to donor cells is expressed as a fraction of his/her

inflammatory response to mismatched PBL, also known as third-party. The mismatched PBL or third-party PBL are antigenically dissimilar cells obtained from normal human subjects. This fraction or ratio is termed the **immunoreactivity index (**IR) (3, 4-6). If the donor-induced response exceeds the response to third-party, the individual is at increased risk for rejection. If the donor response is exceeded by the response to third-party, the individual is at decreased risk. This use of the response to mismatched PBL as a reference response makes test results specific for the transplant recipient and comparable between recipients.

- The immunoreactivity index of the recipient PBL sampled before or after small bowel or liver transplantation has strong correlation with the risk of acute cellular rejection. Therefore, the IR can be used by physicians as a tool in conjunction with all other clinical and laboratory data to predict the transplant patient's rejection risk level.
- Because cadaveric organs are used to perform pediatric liver or small bowel transplantation, and donor cells are consumed during tissue typing, these cells are not available for follow up monitoring of rejection-risk. Therefore, donor-like cells are used to perform this test during extended post-transplant follow-up. These donor-like cells, also known as "surrogate" donor cells are PBL from normal human subjects, who are HLA-matched to the donor at a minimum of one antigen each at the HLA-A-, -B, and -DR loci (5-7). Alternatively, the surrogate donor can have a minimum of one antigen match at the HLA-DR locus, and 2-antigen match at the HLA-A or HLA-B loci. The mismatched PBL are HLA-mismatched to the donor and recipient at all six HLA antigens. Testing results presented in subsequent sections show that rejection-risk assignment, "increased" or "decreased" is not altered by the use of "surrogate" donor cells as stimulators, when compared with actual donor cells.

In the **Pleximmune**^{TM} test system, there are four cell culture reactions as follows:

- **negative control**
  The recipient PBL are cultured *alone* in culture medium *which does not contain* fluorochrome-labeled antibody to the inflammatory response marker, CD154. This group of cells serves as negative control for the Flow Cytometry measurement.
- **Background**
  The recipient PBL are cultured *alone* in culture medium which contains fluorochrome-labeled antibody to the inflammatory response marker, CD154. This group of cells serves as background control for the Flow Cytometry measurement of the background signal caused by the $CD154^{+}$TcM cells present in the unstimulated recipient blood at the time of testing.
- **Donor reaction**
  The recipient PBLs are cultured *with donor or surrogate donor* PBL in culture medium which contains fluorochrome-labeled antibody to the inflammatory response marker, CD154. This group of cells represents the immune reaction of the recipient to the donor.
- **Third-party reaction**
  The recipient PBL are cultured *with mismatched* PBL in culture medium described in the same condition. This group of cells represents the immune reaction of the

recipient to mismatched PBL. This reaction is used as a reference reaction for calculating the IR of the recipient.

Biological Principles of the Assay

Organ transplantation rejection is caused by the transplant recipient's inflammatory immune response to the particular transplanted donor organ (7). The immune response to transplanted organ consists of both cellular (lymphocyte mediated) and humoral (antibody mediated) mechanism. This response begins when the donor organ is recognized as foreign on the basis of antigenic dissimilarity. The inflammatory damage to the transplanted organ is mediated by white blood cells called cytotoxic T-lymphocytes. This inflammatory response requires T-cells to receive two signals from antigen presenting cells, which are among the first cells to recognize foreign antigens (7). Examples of antigen presenting cells are B-lymphocytes, monocytes and dendritic cells. The first signal consists of the foreign antigen presented by the antigen-presenting cell to the T-cell receptor. The second signal consists of a co-stimulatory signal, which is mediated by binding of antigen-presenting cells to the T-cell via a variety of receptor-ligand pairs. Examples include the receptor ligand pairs, CD28: B7, CD40: CD154, etc (8, 9). T-cells which have received both signals damage the transplanted organ directly by secreting cytotoxic substances, e.g. interferon gamma, perforin, etc. The ability to respond to a particular foreign antigen becomes more efficient as the T-cell encounters the antigen repeatedly. Efficient antigen-specific response is a unique feature of "memory" T-cells (10). Direct evidence for the role of CD154 as a mediator of rejection includes the fact that in several experimental models, rejection is attenuated with the use of anti-CD154 antibodies (11). Direct evidence for the role of T-cytotoxic memory cells (TcM) in rejection comes from large animal models in which tolerance or the rejection-free state with no immunosuppression is only seen after ablation of TcM (12).

Warnings and Precautions

- For *in vitro* diagnostic use.
- Pleximmune™ is a qualitative prognostic test that predicts the risk of transplant rejection as either decreased or increased risk of rejection. Quantitative interpretation of the numerical IR value as related to the risk of rejection should not be made.
- The performance of Pleximmune™ during ischemia-perfusion injury, infections such as CMV and EBV, which are opportunistic infections seen in children with liver or small bowel transplantation and graft-versus-host disease is not known.
- Patient blood samples should be collected according to approved Laboratory procedures and processes. Handle all blood samples as potentially infectious according to universal precautions and good clinical laboratory practices.

Ordering Pleximmune™

Pleximmune™ is only performed at Plexision's laboratory. The test can be ordered via Plexision's website http://www.plexision.com/ or calling 1-855-PLEXISION.

Please fill the Test Requisition form (www.plexision.com/Pleximmune-product/ordering information) before shipping the patient sample to Plexision for testing.

Specimen Collection and Shipping Procedure

The following recommendation for specimen collection, shipping and storage of blood samples are based on the Clinical and Laboratory Standards Institute (CLSI) and are augmented with additional sample handling information specific to **Pleximmune™**:

1. Handle all blood samples as if samples are capable of transmitting disease.
2. Whole blood should be obtained by a trained phlebotomist in a hospital-based or a reference laboratory.
3. Collect the whole blood in sodium heparin tubes (green top) only. The minimum whole blood volume requirement for **Pleximmune™** is 3 milliliters (3 mL). The optimum whole blood volume is 5 milliliters (5mL). Any sample which is less than 3mL, Plexision will not perform the test and will immediately notify the physician who ordered the test.
4. Samples should be drawn between the hours of 5:00am and 10:00am. This time frame increases the chances that the sample will arrive at Plexision during normal operating hours when staff is available to test it immediately. Sample collection time and date must be noted on the sample label and the Pleximmune™ Test Requisition Form or the sample will not be accepted.
5. After the blood is collected, it should be inverted gently 5 times to ensure mixing with the sodium heparin. Blood sample with clots or broken seals should be discarded.
6. Plexision will only perform **Pleximmune™** on sodium heparinized whole blood. Blood samples collected in other anticoagulants will not be accepted for **Pleximmune™** testing.
7. To permit identification of a specimen, the specimen label must have at least two patient identifiers, (e.g. date of birth, patient unique identifier assigned by collection center/hospital, name, etc).
8. Complete the **Pleximmune™** Test Requisition Form accessible on the Plexision web site www.Plexision.com. The Test Requisition Form is under Pleximmune/Ordering information. Complete the form by filling in serologic or molecular HLA typing results for donor and patient at HLA-A, HLA-B, and HLA-DR antigenic loci. Confirm that the patient information on the form matches the information provided on the specimen label.
9. Fax a copy of the requisition form to Plexision at (412) 224- 2276 by 4:00 PM (EST) on the day of the shipment.
10. Patient blood sample should be shipped to Plexision's laboratory overnight via FEDEX or UPS at ambient temperature and humidity. Package and label the samples in compliance with applicable federal regulations covering the transport of clinical samples and etiological agents. Samples maintained at room temperature for up to 30 hours are acceptable for testing. Blood samples will be tested as soon as possible after arrival.
11. Blood samples will be discarded if the date and time of sample collection is not noted on the requisition form or label, the container shows frozen or clotted blood, if seals are broken, if the sample is collected in tubes other than sodium heparin tubes, sample is unlabeled, or the sample is not accompanied by either the test requisition form or HLA

information for donor(s) and recipient, or the sample is received beyond 30 hours after the collection time.

12. Patient blood specimen must be accompanied by serologic or molecular typing information at the HLA-A, HLA-B, and HLA-DR antigenic loci for donor(s) and recipient.

Test Procedure

Pleximmune™ is conducted in Plexision's CLIA-certified laboratory. The test process is described in the following steps:

1. Collect patient blood or blood from normal human subjects.
2. Isolate PBLs from patient blood or blood from normal human subjects.
3. Select "surrogate donor PBL" and "mismatched third-party PBL" according to the pre-set selection criteria based on HLA-matching and mismatching as described above.
4. Set up *in vitro* cell culture system to elicit the immune reaction and labeling of CD154+T-cytotoxic memory cells.
5. Measure the frequency of CD154+T-cytotoxic memory cells by flow cytometry.
6. Report and interpret flow cytometry results.
7. Calculate IR and assign corresponding risk of rejection for the patient sample.

Interpretation of Results

How should the results be used?

Pleximmune™ is a prognostic test. It should not be used by itself for any type of clinical decision-making. It should be used in conjunction with biopsy results and the results of clinical and laboratory evaluation to enhance clinical judgment regarding the risk of rejection. During its evaluation, increased or decreased risk of acute cellular rejection measured by Pleximmune™ have been associated respectively with rejection or non-rejection outcomes within a 60-day period after the test (see Clinical Study section below).

How are results presented?

- The test results are provided to the physician as a report. In the report, test results are expressed as an **immunoreactivity index (IR).** The immunoreactivity index or IR, is the ratio of donor- and third-party-induced %CD154+TcM. This analyte is measured by flow cytometry using five fluorochrome-labeled antibodies and a viability dye. The IR is calculated by dividing the count of CD154+TcM induced in the donor reaction by those induced in the third-party reaction.
- **For post-transplant blood samples**, an IR $\geq$ 1.1 implies increased risk. An IR < 1.1 implies decreased risk.
- **For pre-transplant samples**, and IR $\geq$ 1.23 implies increased risk. An IR <1.23 implies decreased risk.

- The number of CD154+TcM per TcM in the donor and third-party reactions are each compared with those present in the background reaction using Poisson's test. At least one reaction must pass the Poisson test ($p \leq 0.05$) for the Pleximmune™ results to be used for rejection-risk measurement. If both reactions fail Poisson test, the test is considered invalid, and no immunoreactivity index is reported. **Please note that** the immunoreactivity index is a calculated absolute numeric value with no units. IR values have ranged from 0.01 to 7.1 in validation studies. Increasing IR values above the rejection-risk threshold should not be interpreted as indicating increasing severity of rejection.

Limitations of the Assay

- **Pleximmune™** must be performed on whole blood collected and shipped as described under specimen collection and shipping procedure above to obtain accurate results.
- Plexision will only perform Pleximmune™ on sodium heparinized whole blood. Blood samples collected in other anticoagulants will not be accepted for Pleximmune™ testing.
- The prediction of increased or decreased risk of rejection provided by the Pleximmune™ is applicable to the 60 day period after the test. The prediction of decreased risk of rejection for the 60 day period after the test does not rule out the possibility of rejection at a later date. Any test result must therefore be interpreted as an adjunct to clinical information.
- Pleximmune™ has not been evaluated in predicting the risk of rejection in samples obtained during days 61-199 after transplantation.
- Pleximmune™ is not approved for use to predict the risk of rejection of organs other than the liver or small bowel in children less than 21 years old.
- Pleximmune™ is not approved for use to predict the risk of rejection in adult transplant recipients.
- Co-infection with viruses and bacteria may affect cell function. Little is known about the performance of Pleximmune™ during infections such as CMV and EBV, which are opportunistic infections seen in children with liver or small bowel transplantation.
- Pleximmune™ performance may be affected by gender- and ethnicity-related effects. However, the sample size limitations imposed by the rarity of the clinical condition addressed by Pleximmune™ (i.e., solid organ transplantation in pediatric patients) did not allow for a comprehensive evaluation of possible gender- and ethnicity-related effects on the test's performance.

Performance Characteristics

Precision study:

- **Saturating concentration** of each fluorochrome-labeled antibody was determined using PBL from normal controls. Saturating concentration was the minimum concentration to detect the highest percentage of positive cells. Fluorochromes and the antibodies to which they are attached are anti-CD3-FITC (fluorescein isothiocyanate), anti-CD8-PE-Cy7 (allophycocyanin-cyanin-7), anti-CD8-APCH7

(allophycocyanin-H7), anti-CD45RO-APC (allophycocyanin), anti-CD154-PE (phycoerythrin), and 7-aminoactinomycin-D (7-AAD).

- **The specificity of each antibody** in the cocktail was demonstrated by measuring the variation in frequencies of CD8+ cells upon adding each antibody alone and in combination with others. The frequency of CD8+ cells or Tc demonstrated a coefficient of variation (%CV) of 3.5%-12.2% in PBL from three normal controls with successive addition of each antibody except anti-CD154-PE. The acceptable maximum %CV for this and subsequent phases of precision testing is 20%.
- **This variation** in the frequency of Tc ranged from %CV 1.04-5.9% when anti-CD154-PE was added to the remaining fluorochrome-labeled antibodies.
- **Lot-to-lot variation** was evaluated between two lots of antibody reagents (13). Lots were compared for their ability to detect the frequency of cells expressing their respective target markers using PBL from three normal control assays performed in duplicate. The %CV ranged from 0.9-15.3%.
- **Within-run and between-run variation:** Same-day variation in %CD154+TcM was evaluated between duplicate assays (a and b) within and between each of two runs (1 and 2). Assays were performed between HLA-mismatched responder-stimulator PBL from normal human subjects. Summary for background %CD154+TcM and stimulated %CD154+TcM for 20 such assays and is shown in Table 1 below.

Table 1: %CD145+TcM for 20 duplicate assays (a and b) in each of two runs, (1 and 2)

Run	Reaction	N	Mean	CI-low	CI-up	SD	Median	Min	Max
1a	Background	20	0.3	0.18	0.51	0.35	0.25	0	1.6
	Stimulated	20	24.1	19.0	29.2	10.8	25.0	3.7	41.2
1b	Background	20	0.3	0.19	0.42	0.25	0.3	0	1.1
	Stimulated	20	24.2	19.0	29.4	11.1	24.6	3	41.3
2a	Background	20	0.28	0.12	0.44	0.35	0.2	0	1.5
	Stimulated	20	25.1	19.6	30.5	11.6	25.2	2.9	42.5
2b	Background	20	0.24	0.13	0.36	0.25	0.2	0	1.1
	Stimulated	20	25.2	19.9	30.4	11.3	26.4	3.2	43.1

*CI=95% confidence interval, low = lower bound of CI, up = upper bound of CI

- The source of variation from with-in runs, between runs, and between samples is shown in Table 2.

Table 2: Source of Variability in Precision Study

Measure/ Type of Variation	Variation	% of Variation	SD
Background %			
Within Runs	0.0176	15.9%	0.1327
Between Runs	0.0152	13.7%	0.1233
Between Samples	0.0778	70.3%	0.2790
Total Variation	0.1107		0.3327
Stimulated %			
Within Runs	2.1	1.7%	1.46
Between Runs	1.3	1.0%	1.14
Between Samples	124.0	97.3%	11.14
Total Variation	127.5		11.29

- A normal range for background and stimulated %CD154 was calculated based on the overall mean +/- $T_{0.975,19}$*SD obtained from using the estimated variability between the samples shown in Table 3. **A reference range** for allospecific CD154+TcM induced in PBL from 20 normal human subjects is **24.6 +/- (1.96*11.1), or 2.8 to 46.4,** consistent with the observed minimum and maximum values observed in the data. The precision analyses supported the repeatability of the test. Note that the between-sample variance accounted for 98.1% of the variability in the sample. The within-run SD estimate was 1.46. The variability between samples provided a lower percentage among background samples (74.5%), but between subject variability is expected to be low in this setting.
- Table 3 summarizes the %CV for duplicates within runs and between runs.

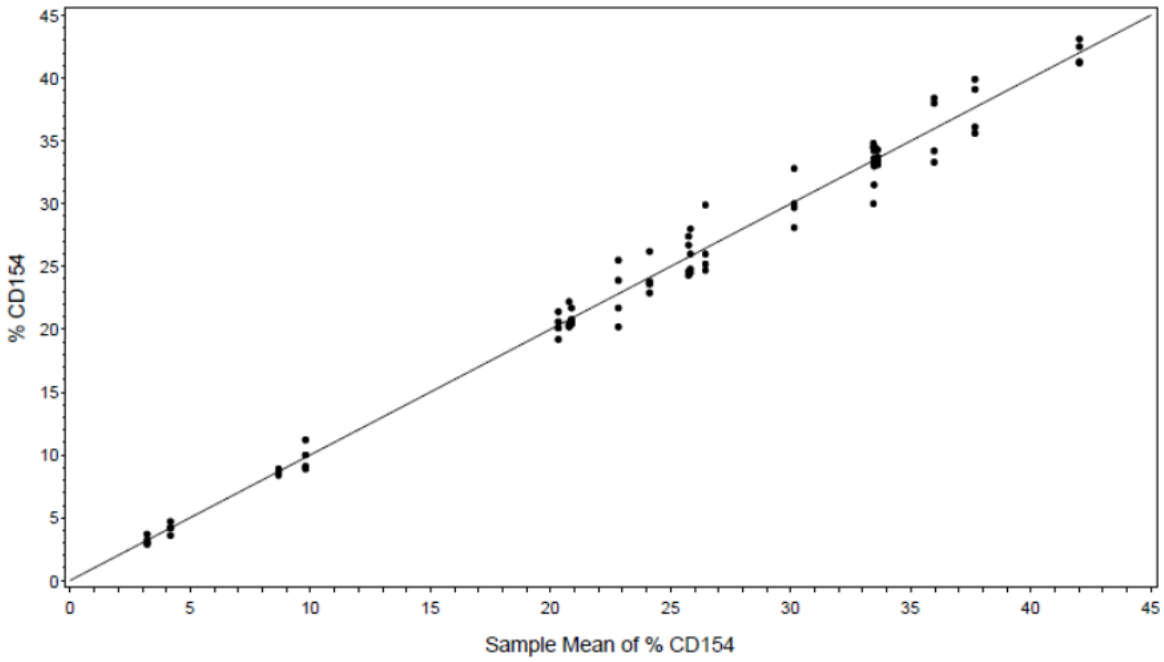
Table 3: %CV for %CD154+TcM within each of two runs, 1 and 2, and for all replicates performed in both runs

Run	Reaction	N	Mean	CI-low	CI-up	SD	Median	Min	Max
1a & 1b	Background	20	38.9	17.0	60.9	47.0	27.2	0.0	142.0
	Stimulated	20	5.2	2.9	7.4	4.9	4.0	0.2	16.4
2a & 2b	Background	19	43.0	20.1	65.9	47.5	28.4	0.0	142.0
	Stimulated	20	5.4	3.5	7.2	4.0	5.9	0.6	16.2
1a-2b	Background	20	61.3	42.9	79.7	39.3	54.7	18.2	200.0
	Stimulated	20	6.0	4.6	7.5	3.1	6.0	1.5	11.1

*CI=95% confidence interval, low = lower bound of CI, up = upper bound of CI

- The mean (SD) %CV for duplicates within runs and for all replicates in both runs ranges from 5.2 (4.9)% to 6.0 (3.1)%. For the background reaction, %CV are higher, ranging from 38.9 (47.2)% to 61.3 (39.3)%, consistent with the lower finding that between sampling variance is a higher proportion of the results in stimulated samples.
- In order to evaluate the relative variability of the data by value, individual values were plotted against the overall mean of the samples (See Figure 1).

Figure 1: Observed % Stimulated CD154 Values by Sample Mean for the Precision Data



- Figure 1 indicates that the variability of the data with the mean value which is an expected result based on the average %CV associated with pairs of samples.

- **Three-operator, three instrument (flow cytometer) precision testing was done to** evaluate the variability of the test measure across three operators using three copies of the instrumentation. Mean and median frequencies (%) of allospecific CD154+TcM, and variation in these frequencies between three-operators in same-day testing are shown in Table 4.

Table 4: %CD154+TcM on same day by three technologists/instruments (1a, 1b, 1c)

Run	Reaction	N	Mean	CI-low	CI-up	SD	Median	Min	Max
1a	Background	21	4.0	2.3	5.7	3.8	2.9	0.5	13.2
	Stimulated	21	24.5	19.5	29.6	11.0	24.7	9.0	55.0
1b	Background	21	5.0	2.8	7.2	4.9	3.5	0.9	19.2
	Stimulated	21	22.8	18.0	27.6	10.5	22.4	9.8	52.2
1c	Background	21	5.8	3.2	8.3	5.7	3.4	1.2	18.4
	Stimulated	21	22.9	18.0	27.8	10.8	22.4	9.8	51.7

*CI=95% confidence interval, low = lower bound of CI, up = upper bound of CI

- The proportion of variance attributable to the operator/instrumentation set and between samples is provided in Table 5 for same-day testing by three-operators-instruments.

Table 5: Analysis of variance associated with operators/instrumentation and between samples

Measure/ Type of Variation	Variation	% of Variation	SD
Background %			
Between Operators	4.54	17.8%	2.13
Between Samples	21.01	82.2%	4.58
Total Variation	25.55		5.05
Stimulated %			
Between Operator	3.69	3.1%	1.92
Between Samples	114.30	96.9%	10.69
Total Variation	117.99		10.86

- The mean (SD) background CD154+TcM ranged from 4.0 (3.8)% to 5.8 (5.7) % in same-day testing of 21 samples. For the stimulated reaction, mean CD154+TcM ranged from the mean (SD) 22.9 (10.8)% to 24.5 (11.0) % in same day testing. In the stimulated samples, the proportion of variance relating to operators was 3.1% for same day testing.
- **An annual proficiency/precision study** among three flow cytometry technicians was conducted (Table 6). A %CV of 3.3 to 8.05% for test results on the same sample was below the acceptable maximum %CV of 20%.

Table 6: % CD154+TcM measured by three technologists in each of three responder PBL

Un-blinded code	Blinded code	Tech ID	% CD154+TcM	Mean	CV %
N071	UK01	2	4.7	4.86	4.3
	UK03	1	4.8		
	UK05	3	5.1		
N026	UK02	2	5.5	5.16	8.05
	UK04	1	4.7		
	UK06	3	5.3		
N041	UK07	2	2.9	3.0	3.3
	UK08	1	3.1		
	UK09	3	3.0		

Operator to operator variation:

Background: Test results for the same sample can vary between multiple technicians.

Goals: This study provides a measure of variation between two technicians for the same sample.

Methods: CLIA-approved proficiency testing describes this type of evaluation (SOP15-02). The same blinded sample was tested by two different operators on the same day, and %CV determined for %CD154 in background and stimulated reactions.

Number of samples tested: Five samples.

Results: %CD154+TcM in each sample, variation between technicians for each sample, mean %CD154+TcM for all samples for each technician, and mean %CV between technicians for all five samples tested are shown in Table 7 - Table 10 below.

Table 7: %CD154+TcM in background and stimulation reactions for each sample for each of two technicians (Tech 1 and Tech 2)

			Background	Stimulated
Tech	Unblinded code	Blinded code	%CD154+TcM	%CD154+TcM
Tech 1	N071	UK03	0.4	4.8
Tech 2		UK05	0.1	5.1
Tech 1	N026	UK04	1.6	4.7
Tech 2		UK06	0.6	5.3
Tech 1	N041	UK08	1.1	3.1
Tech 2		UK09	0.7	3

			Background	Stimulated
Tech 1	N040	UK01	1.7	7.4
Tech 2		UK03	0.5	8.2
Tech 1	N014	UK02	1.3	7.8
Tech 2		UK04	1.7	7.6

Table 8: Variation in %CD154+TcM between technicians for each sample

		Background			Stimulated		
Sample	Tech	mean	SD	%CV	mean	SD	%CV
N071	Tech 1 vs 2	0.25	0.21	84.0	4.95	0.21	4.2
N026	Tech 1 vs 2	1.10	0.71	64.5	5.00	0.42	8.4
N141	Tech 1 vs 2	0.90	0.28	31.1	3.05	0.07	2.3
N040	Tech 1 vs 2	1.10	0.85	77.3	7.80	0.57	7.3
N014	Tech 1 vs 2	1.50	0.28	18.7	7.70	0.14	1.8

Table 9: Mean %CD154+TcM for all samples for each of two technicians

Tech	Reaction	N	Mean	CI-low	CI-up	SD	Median	Min	Max
Tech 1	Background	5	1.2	0.6	1.9	0.5	1.3	0.4	1.7
	Stimulated	5	5.6	3.1	8.0	2.0	4.8	3.1	7.8
Tech 2	Background	5	0.7	0	1.5	0.6	0.6	0.1	1.7
	Stimulated	5	5.8	3.2	8.4	2.1	5.3	3.0	8.2

Table 10: Mean %CV for %CD154+TcM for all samples for each of two technicians

Tech	Reaction	N	Mean	CI-low	CI-up	SD	Median	Min	Max
Tech 1 and 2	Background	5	55.1	19.4	90.9	28.8	64.5	18.7	84.0
	Stimulated	5	4.8	1.1	8.5	3.0	4.2	1.8	8.4

Conclusions: Variation in %CD154+TcM in stimulation reaction is acceptable at 4.8% for five samples tested by two technicians, and below the pre-specified threshold of %CV <20%.

Day-to-day variation:

- Samples are expected to arrive at Plexision in a manner that allows for testing on the day of sampling if received before 10AM, or the day after sampling if received after 10AM on day of sampling or after overnight shipment. Therefore, it is necessary to understand variation in sample results after 24 hour storage in Plexision's lab or after an overnight shipment.
- Each of five samples from normal human subjects was divided into three aliquots for same day testing (1a), testing after 24-hour storage at ambient temperature in Plexision's laboratory (1b), and testing after overnight shipment at ambient temperature (1c). The same technician tested all three aliquots of a given sample. A single cytometrist acquired results by flow cytometry.
- Mean %CD154+TcM for all samples in each of three storage conditions, and mean %CV between the three storage conditions is shown in the two tables (Table 11 and Table 12) below.
- Day-to-day variation in %CD154+TcM in stimulated reaction for a sample tested under three different conditions of storage was acceptable at mean %CV of 3.2%, and below the pre-specified threshold of %CV <20%.

Table 11: Mean %CD154+TcM for each condition of storage/shipment of five sample in day to day variation testing

Storage condition	Reaction	N	Mean	CI-low	CI-up	SD	Median	Min	Max
1a (same day)	Background	5	2.1	-0.7	4.9	2.3	2	0.1	5.8
	Stimulated	5	5.5	-1.3	12.3	5.5	3.9	1.4	14.6
1b(24h, ambient temp)	Background	5	1.6	-1.3	4.5	2.3	0.6	0.3	5.7
	Stimulated	5	5.7	-1.3	12.7	5.6	4.4	1.4	15.1
1c (24 hr, overnite shipment)	Background	5	2.3	-0.2	4.8	2	2.9	0.1	3.7
	Stimulated	5	5.5	-1.1	12.2	5.4	4.1	1.4	14.5

Table 12: %CV for %CD154+TcM between three conditions of storage/shipment for five samples

Storage condition	Reaction	N	Mean	CI-low	CI-up	SD	Median	Min	Max
1a, 1b and 1c	Background	5	60.3	22.4	98.2	30.5	66.7	7.9	86.6
	Stimulated	5	3.2	-0.6	7.0	3.0	2.2	0	6.7

Variation due to cryopreservation:

Pleximmune™ has been evaluated in patient samples cryopreserved at temperatures below minus 75 degrees Centigrade. To determine whether cryopreservation affected cell function, which is the basis for rejection-risk assessment, a study was conducted. Because such studies require large amounts of blood sample, which cannot be obtained from small pediatric patients, a study was performed with normal human blood samples.

Methods: Cell function was measured under the most ideal (fresh stimulator and responder) and the least ideal (cryopreserved stimulator and responder) combination of stimulator and responder cells. Cell function was measured by %CD154+TcM. The frequency of CD154+TcM induced by stimulation with HLA-non-identical PBL, was compared in samples tested before and after 30 days of cryopreservation. Fresh stimulator and responder cells were used in samples tested before cryopreservation. Cryopreserved stimulator and responder cells were used in samples tested after 30 days or cryopreservation.

Number of samples tested: 20

Results: Mean %CD154+TcM before and after cryopreservation in all samples tested, and mean %CV for all samples tested in shown in tables below.

Conclusions: The results suggest that the variation in the stimulated %CD154+TcM measured as mean %CV before and after 30-day cryopreservation was acceptable at 8.9%, and below the pre-specified threshold of <20%, as shown in Table 13 and Table 14.

Table 13: Mean %CD154+TcM in 20 normal human blood samples tested before and after 30-day cryopreservation

sample type	Reaction	N	Mean	CI-low	CI-up	SD	Median	Min	Max
Fresh	Background	20	4.2	2.4	5.9	3.8	3	0.6	13.2
	Stimulated	20	24.8	19.5	30	11.3	25.2	9	55
30-day cryo	Background	20	6.1	4.1	8.2	4.4	4.7	1.1	17
	Stimulated	20	22.8	17.7	27.9	10.9	23.2	9.1	52.6

Table 14: Mean %CV for CD154+TcM in 20 normal human blood samples tested before and after 30-day cryopreservation

Sample type	Reaction	N	Mean	CI-low	CI-up	SD	Median	Min	Max
Fresh vs 30-day cryo	Background	20	45.3	33.8	56.8	24.5	42.6	5	87.2
	Stimulated	20	8.9	5.6	12.2	7	6.5	0.8	24.9

Clinical study

Validation samples (n=122, 87 subjects) were collected as a component of an open-label, single-center, observational research protocol at Children's Hospital of Pittsburgh (CHP), were, de-identified, and tested at Plexision. Demographics and sample characteristics for the validation set are shown in Table 15.

Table 15: Subject and sample characteristics

	Validation set (March 19, 2009 to August 16, 2012)	
Parameter	**All**	**Analyzable**
Total subjects	87	72
Subject Age (Years) (mean +/-SD, range)	8.6±6.7, 0.4 to 20.2	8.7±6.6, 0.4 to 20.1
Gender (M:F)	48:39	41:31
Race (Caucasian: non-Caucasian)	68:19	55:17
Organ (L: LSB: SB: LK: LL)	71:5:10:0:1	61:2:8:0:1
Induction (None: thymo: campath)	38:46:3	32:37:3
FKWB (ng / ml)	7.9 ± 5.1	7.5 ± 4.6
Actual donor: surrogate donor	6 : 81	5 : 67
Total samples	122	97
IR0 samples	43	33
IR1 samples	39	30
IRx samples	40	34
Time between Tx and sample (days) IR0 / (range)	-4.1 ±15.6 (-85 to 0)	-5.2 ±17.7 (-85 to 0)
Time between Tx and sample (days) IR1+IRx	938± 1435 (6 to 5360)	989 ± 1474 (6 to 5360)
Time between sample and biopsy/event (days) / (range)	15.2±17.2 (0 to 59)	13.8±17.0 (0 to 59)
Failure to generate signal	16	
Inadequate viable cell counts	9	

From 87 subjects, 122 pre- and post-transplant samples were blinded and tested with Pleximmune™ in Plexision's laboratory. All samples contained at least 2 million peripheral blood lymphocytes. Samples containing at least 0.45 million viable lymphocytes were evaluated with Pleximmune™. Fewer viable cells led to the sample being discarded for insufficient cells (Table 15). Those samples, which failed to demonstrate significant increase in both the donor- and third-party-induced CD154+TcM on Poisson test were discarded (Table 15).

Using the post-transplant rejection-risk cutoff threshold of 1.10, the sensitivity, specificity, PPV and NPV were 84%, 80%, 64% and 92%, respectively (AUC 0.791) when applied to 64 post-transplant samples in the validation study. Using the pre-transplant rejection-risk cutoff threshold of 1.23, the sensitivity, specificity, PPV and NPV were 57%, 89%, 80%, 74%, respectively (AUC 0.842), when applied to 33 IR0 samples in the validation study. The results are shown in Table 16.

Table 16: Validation Study Results

Cohort	AUC	Sensitivity [n] (95% CI)	Specificity [n] (95% CI)	PPV [n] (95% CI)	NPV [n] (95% CI)
IR0 set (n=33)	0.842	**57%** [8/14] (30% - 81%)	**89%** [17/19] (65% - 98%)	**80%** [8/10] (44% - 96%)	**74%** [17/23] (51% - 89%)
IR1+IRx set (n=64)	0.791	**84%** [16/19] (60% - 96%)	**80%** [36/45] (65% - 90%)	**64%** [16/25] (43% - 81%)	**92%** [36/39] (78% - 98%)

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Requisition Form: Pleximmune™

Patient Name:

Date of Birth: **Gender:** ◯ Male ◯ Female

Insurance Details:

Carrier: Plan Name:

Member ID #: Group ID #:

Additional Comments, if any:

Sample Collection Date: **Time:** ◯ AM ◯ PM

Timing of sample: ◯ Pre-transplant ◯ Post-transplant

Sample Volume: ◯ 3-5 ml ◯ Other

(Five ml is the preferred sample volume. Samples less than 3 ml may be discarded because results may be inaccurate).

Sample Container: Sodium Heparin (green top) ☐

Shipping Conditions: Ambient Temperature ☐

Shipping time (from phlebotomy to delivery): ◯ <30 hours ◯ >30 hours

Patient HLA: HLA-A: HLA-B: HLA-DR:

Donor HLA: HLA-A: HLA-B: HLA-DR:

(Samples without accompanying HLA information for patient and donor will not be tested).

Physician Ordering Test:

NPI #:

Facility: **Facility Phone:**

Facility Fax:

Ship To: Plexision
4424 Penn Avenue, Suite 202, Medical Building
Pittsburgh, PA 15224
Phone: 1(855)-753-9474 or 1(855)-PLEXISI; Fax: 412-224-2776

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State ID: 32485 CLIA ID: 39D2042664

Test Report : Pleximmune™

Patient Name: **Accession Number:**

Insurance Details:

Carrier: Plan Name:

Member ID #: Group ID #:

Additional Comments, if any:

Date of Birth: **Gender:** ◯ Male ◯ Female

Sample Collection Date: **Time:** ◯ AM ◯ PM

Sample Receipt Date: **Time:** ◯ AM ◯ PM

Time from transplant: Pre-transplant ☐ **Post-transplant** ☐

Sample Condition when received:

Sodium Heparin Tube: ◯ Yes ◯ No Seal Intact: ◯ Yes ◯ No

Blood Clot: ◯ Yes ◯ No Volume: ◯ 3-5 ml ◯ Other

Recipient and donor HLA information: ◯ Yes ◯ No

Sample: ◯ Not adequate for assay ◯ Assayed ◯ Failure to generate signal

Patient HLA: HLA-A: HLA-B: HLA-DR:

Donor HLA: HLA-A: HLA-B: HLA-DR:

Physician Ordering Test: **NPI #:**

Facility:

Assay Report Date: **Time:** ◯ AM ◯ PM

Donor-induced CD154+TcM: % **Third-party-induced CD154+TcM:** %

Immunoreactivity Index (IR):

Assay interpretation: Increased/Decreased risk of rejection

Reference range: For post-transplant blood samples, an IR >= 1.1 implies increased risk. An IR < 1.1 implies decreased risk.
For pre-transplant samples, an IR >= 1.23 implies increased risk. An IR <1.23 implies decreased risk.

Interpretation: Results of this assay should be used in conjunction with medical history, clinical presentation and other clinical and laboratory indicators when establishing the risk of rejection. The risk of rejection is specific to the transplant recipient. The immunoreactivity index is a calculated numeric value. The actual value does not indicate the severity of rejection. The value only indicates increased or decreased risk of rejection based on whether it is at or above the rejection thresholds as described under reference range.

Humanitarian Device. Authorized by Federal Law for use as an aid in the evaluation of the immunological risk for Acute Cellular Rejection (ACR) in children with liver or small bowel transplantation. The effectiveness of this device for this use has not been demonstrated.

Limitation: Pleximmune™ is an FDA-approved In Vitro Diagnostic test developed and performed by Plexision. Test results should be used as an adjunct to clinical information and laboratory results to aid in the evaluation of the immunological risk for Acute Cellular Rejection (ACR) in children with liver and small bowel transplantation. The Pleximmune™ test has neither prognostic nor diagnostic value for the evaluation of Antibody Mediated Rejection (AMR), nor Chronic Rejection. The performance of the test has not been established in the presence of viral or bacterial infections, or during the period of 61-199 days after transplantation.

Director:

Name: Signature: Date & Time:

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