Protocol with Statistical Analysis Plan Cover Page:

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Title: Red Cell Half Life Determination in Patients with and without Sickle Cell Disease

Abbreviated Title: Red Cell Survival

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Other identifying words: Red cell survival, cohort label, biotin, sickle cell anemia, sickle cell disease

Principal Investigator: John F. Tisdale, MD, CMTB, NHLBI (E)

Estimated duration of study: 18 months

Subjects of study:	Number	Sex	Age range
Patients with Sickle Cell Disease (SCD) and controls	21*	either	18 and above

*(*The accrual goal is 21 evaluable subjects; however, to cover screenfails, withdrawal, drop-outs and loss-to-follow ups, an accrual ceiling of 40 subjects is allowed).*

Cohorts
- SCD pre-transplantation
- SCD post-transplantation
- HbAS
- HbAA (Healthy volunteers)

Project involves ionizing radiation?	No
Project uses "Durable Power of Attorney"?	No
Offsite project?	Yes
Multi-institutional project?	No

Investigational Agent:

Drug Name:	CC TAG BIOTIN ®
IND Number:	IND 19550
Sponsor:	NHLBI OCD
Manufacturer:	ChemConnection BV

PROTOCOL SUMMARY

Synopsis

Title:	**Red Cell Half Life Determination in Patients with and without Sickle Cell Disease**
Study Description:	**This study will use biotin-labeling of red blood cells (RBCs) to determine the mean potential lifespan (MPL) of RBCs in patients with sickle cell disease (SCD) compared to patients who have successfully undergone curative bone marrow transplantation (BMT, allogeneic or autologous), participants with sickle cell trait, and healthy donors without SCD. Previous studies have corroborated the MPL of healthy donor RBCs to be approximately 115 days while RBCs from patients with SCD have a much more variable but consistently shorter MPL of approximately 32 days. Allogeneic BMT is a curative therapy for the treatment of severe SCD with stable, mixed donor–recipient chimerism after BMT sufficient to reverse the sickle cell phenotype by virtue of improved donor red cell survival compared to the ineffective erythropoiesis of SCD. We predict that the hematologic variables associated with red cell survival among patients with SCD vs. participants with SCT and healthy donors can be used to determine the necessary amount of corrected hemoglobin required to overcome the red cell pathology of SCD. Data generated will be used to determine the utility of performing a population study of RBC lifespan in gene therapy treated patients to ultimately target the percentage of transferred globin gene needed to reverse SCD. The data generated will refine our understanding of the degree of correction necessary to reverse the phenotype of SCD.**
Objectives:	**Primary Objective: To determine and compare red blood cell survival in patients with SCD, patients with SCD who have undergone BMT, participants with SCT, and healthy donors, and validate the association of red cell survival with known markers of increased red cell survival.**
	Exploratory Objective: To create a mathematical model incorporating RBC survival and reticulocyte count to determine the necessary amount of normal hemoglobin, and therefore vector copy number or amount of transferred globin, required for gene therapy protocols.
Endpoints:	**Primary Endpoint: Red blood cell survival** **Secondary Endpoints: Relationship of red blood cell survival to hematologic parameters. Antibody detection to biotin.**
Study Population:	**Healthy volunteers, participants with known SCT or SCD, and patients with SCD who have successfully undergone curative hematopoietic stem cell transplant. No selection will be based upon gender, race, or age (in those 18 years or greater in age). Participants must be available for frequent blood draws required by and for the duration of this study.**
Phase:	**Pilot**
Description of Sites/Facilities Enrolling Participants:	**This is a pilot single site study. Participants will be enrolled at the Clinical Center at the National Institutes of Health.**
Description of Study Intervention:	**The MPL of RBCs from patients with SCD will be compared to patients who have successfully undergone curative BMT, participants with SCT, and healthy donors without SCD, the latter two who serve as donors for patients with SCD who undergo BMT. RBCs will be isolated**

	from participant whole blood. RBCs will be biotinylated ex vivo and reinfused the following day. Biotin can be easily tracked via flow cytometry therefore venous sampling will occur at regular intervals until the percentage of biotinylated RBCs has decreased to the lower limit of detection.
Study Duration:	**Estimated 18 months**

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STATEMENT OF COMPLIANCE

The trial will be carried out in accordance with International Conference on Harmonisation Good Clinical Practice (ICH GCP) and the following:

- United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46, 21 CFR Part 50, 21 CFR Part 56, and/or 21 CFR Part 312)

National Institutes of Health (NIH)-funded investigators and clinical trial site staff who are responsible for the conduct, management, or oversight of NIH-funded clinical trials have completed Human Subjects Protection and ICH GCP Training.

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the Institutional Review Board (IRB) for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. In addition, all changes to the consent form will be IRB-approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

1.0 Introduction and Background

Sickle cell disease (SCD) is a severe hereditary form of anemia in which a mutated form of the beta globin gene distorts red blood cells and decreases red cell survival. Unstable sickle hemoglobin rapidly polymerizes into gelled, aggregated hemoglobin S (HbS) tetramers at low oxygen levels, leading to increased red cell rigidity, oxidative damage of the red cell membrane, distorted red cell morphology, and a shortened red blood cell (RBC) survival. The altered rheology of erythrocytes and chronic hemolysis leads to the numerous downstream effects including activation of monocytes, leukocytes, platelets, plasma proteins and endothelium. This red cell rigidity and subsequent inflammation is responsible for the devastating clinical manifestations of SCD including chronic anemia, viscosity changes, intravascular sludging, stroke, end organ damage, and ultimately, decreased overall survival (1).

Inherent instability of the HbS polymer leads to oxidative damage of the red cell membrane, with subsequent activation of ion channels leading to a dehydrated and acidotic RBC with increased risk for hemolysis and decreased lifespan. In contrast with the lifespan of RBCs from healthy donors (HD) or even individuals with sickle cell trait (SCT) who have been shown to have an RBC lifespan of approximately 107-115 days (2-4), the mean life span of RBCs in patients with SCD is approximately 10-32 days (2, 5-6)**.** Certain molecular factors are thought to affect the severity of anemia in patients with SCD, likely reflected by different rates of hemolysis and therefore different hemoglobin levels at steady state. Several biochemical markers of hemolysis have a correlation to erythrocyte survival in patients with SCD, including a strong inverse correlation with absolute reticulocyte count (ARC) ($r=-0.84$, $p<0.0001$), percent of reticulocytes ($r=-0.78$, $p=0.002$), α globin gene number ($r=-0.15$, $p=0.008$), and a positive correlation with percent fetal hemoglobin (HbF) ($r=0.58$, $p=0.038$) (2,4). The range of HbF content in fetal hemoglobin containing cells (F cells) appears to be important, with cells on the high end of the range surviving longer than those on the low end (7). Furthermore, the survival of non-F cells correlates inversely with the fraction of F cells, with shorter non-F cell RBC lifespan in patients with a high percentage of F cells independent of hydroxyurea therapy (7).

There are two methods to determine red cell survival; *in vivo* cohort methodology or *in vitro* population labeling. Cohort labeling involves labeling hemoglobin with oral glycine containing radioactive or stable isotopes (^{15}N-glycine) which becomes metabolically incorporated into hemoglobin-synthesizing normoblasts in the bone marrow (BM). The resulting labeled RBCs are similarly aged, and mature and are released from the BM at the same time. The quantification of RBC lifespan would therefore require a full time-course study of approximately 150 days (based on HD data and time to maximum ^{15}N-glycine detection) and analysis by mass spectrometry. While this method allows study of single age cohort and does not require *ex vivo* manipulation, samples are not analyzed until the completion of the study and would therefore not be available live throughout the study.

In contrast to the cohort method, current population labels include biotin and ^{51}Cr, which tag a representative population of circulating RBCs irrespective of RBC age. The ideal label has minimal RBC manipulation, stability throughout the life of the RBC, lack of RBC shortening due to labeling, and lack of toxicity to the individual receiving labeled RBCs. Biotinylated RBCs (BioRBCs) confer advantages over radiolabeled methods by upholding these ideals in addition to posing no radiation risks and providing a more acceptable method to study vulnerable populations such as women, fetuses, infants, and children. A further advantage of the population study method is the label (ie. biotin) can be easily tracked via flow cytometry, and the analysis can be complexed with additional characterization parameters such as intracellular staining for different hemoglobin moieties**.** The labeled population of cells is representative of what is found in circulation at the time of labeling and therefore the RBCs are of different ages. This type of study has allowed for measurement of phosphatidylserine exposure on RBCs as they age in vivo (8) and the quantification of lifespan of F cells versus non-F cells in SCD patients as previously described (7).

Additionally, a retrospective analysis of a population study data set suggests that a 28-day window may be sufficient for accurate quantification of MPL(4).

The only currently available cure for SCD is bone marrow transplantation (BMT), with gene therapy research active and underway. Stable, mixed donor–recipient chimerism after BMT is sufficient to reverse the sickle cell phenotype by virtue of improved donor red cell survival compared to the ineffective erythropoiesis of SCD (9). A previously developed mathematical model to test the hypothesis that the percentage of necessary donor myeloid chimerism is determined solely by the differences between donor and recipient RBC half-lives has been described (10). As stable mixed chimerism is intrinsically linked to the differences in RBC survival in healthy vs. the SCD genotype, understanding the variability and predictors of red cell survival among patients with SCD vs. individuals with SCT and healthy donors is critical for applied work in gene therapy research which seeks to generate sufficient corrected hemoglobin to overcome the red cell pathology of SCD.

Here we described a method to study the MPL of RBCs from patients with SCD, compared to patients who have successfully undergone curative BMT, individuals with SCT, and healthy donors without SCD, the latter two who serve as donors for patients with SCD who undergo BMT. Unlike biotinylation data from patients with SCD and healthy donors, the MPL of RBCs from participants with SCT has only previously been validated using the ^{51}Cr population method (3). As individuals with SCT are valuable donors to patients with SCD undergoing HSCT, validation of the biotinylation method in this important population is critical. Understanding the differences in MPL of RBCs from these groups using a uniform method may ultimately guide gene therapy protocols to determine adequate hemoglobin content, ie. vector copy number (number of lentiviral vector copies carrying the therapeutic gene of interest in individual target cells, VCN) and/or the amount of corrective fetal or adult globin, required to overcome the SCD phenotype as it relates directly to red cell survival. Such application in the gene therapy patients will help target the percentage of transferred globin needed to reverse SCD based on a patient's engraftment, VCN or transferred globin editing, and red cell survival.

2.0 Objectives

2.1 Primary: To determine and compare red blood cell survival in patients with SCD, patients with SCD who have undergone hematopoietic stem cell transplantation, participants with SCT, and healthy donors, and validate the association of red cell survival with known markers of increased red cell survival including ARC, % HbF, and F cell percentage.

2.2 Exploratory: To create a mathematical model incorporating RBC survival and reticulocyte count to determine the necessary amount of normal hemoglobin, and therefore VCN or amount of edited/transferred globin, required for gene therapy protocols. This model can be validated by studying lifespan in patients after gene therapy treatment.

3.0 Scientific and Clinical Justification

The safety of biotinylating RBCs in SCD patients is known as is a shortened RBC survival. Known influencers of RBC survival such as ARC, %F, and F cells, and mixed chimerism after BMT is sufficient to reverse the sickle phenotype by virtue of improved donor red cell survival compared to the ineffective erythropoiesis of SCD. The percentage of transferred globin necessary to cure SCD through a gene therapy approach and the relationship among ARC, amount of HbA and/or HbF, and measured red cell survival is currently unknown. Furthermore, RBC survival in patients with SCD who have successfully undergone HSCT has never been performed, as has the biotin methodology for individuals with SCT who are a critically important donor source for patients undergoing BMT.

Collection and biotinylation of RBCs in this study will allow the measurement and study of red cell survival in patients with and without SCD. The study of RBC survival and its relation to known influencers of red cell survival including ARC, % HbF, and F cell percentage will allow for the creation of a mathematical model that will help determine necessary amount of normal hemoglobin required to reverse the SCD phenotype. Such determination has the potential to influence current and future gene therapy protocols. Future questions include the ability to measure RBC lifespan in gene therapy patients, and the relationship of transferred globin (VCN) and percent engraftment of gene modified cells to the patient's RBC survival. As has been previously described (10), the reticulocyte fraction is a good proxy for RBC lifetime, and therefore gene therapy may be most efficacious in patients in which RBC survival is lowest (with highest reticulocyte count) as they would require lower amounts of normal hemoglobin production (lower levels of gene correction) to sufficiently reverse the SCD phenotype.

The population method will be used here to study RBC lifespan due to the safety, potential for a substantially shorter study period, and the possibility of being able to complex the labeling of the RBCs with other markers of interest.

4.0 Subject Eligibility

4.1 Inclusion criteria:

4.1.1 Age 18 or greater with a confirmed diagnosis of homozygous SCD (HbSS, HbSC, HbSB0), sickle cell trait (HbAS), or healthy volunteer (HbA)
4.1.2 Normal renal function: creatinine <1.5 mg/dL
4.1.3 Negative direct antiglobulin test (DAT)
4.1.4 Ability to give informed consent to participate in the protocol

4.2 Exclusion criteria:

4.2.1 Any uncontrolled chronic illness other than sickle cell disease
4.2.2 Active viral, bacterial, fungal, or parasitic infection
4.2.3 Consumption of biotin supplements or raw eggs within 30 days
4.2.4 Blood loss within the previous 8 weeks >540mL
4.2.5 Pregnancy
4.2.6 Pre-existing, naturally occurring antibodies against biotin

5.0 Study Design and Methods

5.1 Description of Study Population and Recruitment

This is a pilot single site study and participants will be enrolled at the NIH Clinical Center. Recruitment will occur from a pool of normal volunteers, individuals with known SCT or SCD, and patients with SCD who have successfully undergone curative hematopoietic stem cell transplant. Volunteers will be recruited through the Clinical Center Volunteer Office.

The recruitment ceiling is an N=40 to account for screen failures. Accrual will be stopped once the numbers of active participants who have completed data collection have been reached:

- Normal Volunteers: N=3
- Individuals with SCT: N=6
- Individuals with SCD pre-transplantation: N=6
- Individuals with SCD post-transplantation: N=6-10

Accrual will be based solely up on protocol entry criteria and no selection will be based upon gender, race, or age (in those 18 years or greater in age). The study will be listed on the clinicaltrials.gov, Clinical Center research studies, and the National Heart, Lung and Blood Institute patient recruitment websites.

5.2 Study Design

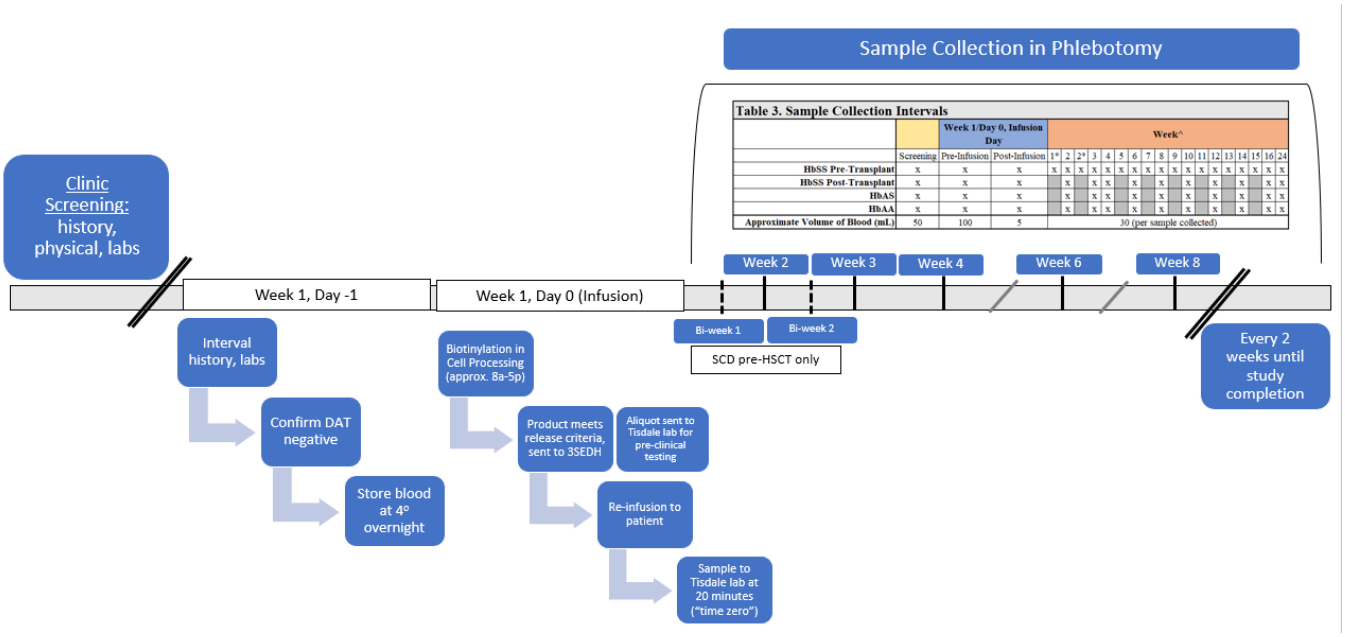

Figure 1. Schematic diagram of the study design
*Participants with SCD pre-HSCT will have samples collected weekly until completion of study.

6.0 Procedures, Blood Collection, Laboratory Tests and Processing

6.1 Screening: Potential and enrolled participants may have had pre-study evaluation procedures conducted for clinical and research purposes under the IRB-approved NHLBI screening protocol 97H0041. Prior, concurrent and subsequent results from pre-study evaluation procedures performed under this screening protocol may be utilized to determine eligibility for this protocol.

If previous pre-study evaluations are not available from 97H0041, then all screening activities will be conducted under this protocol.

First, all volunteers will be evaluated with a medical history and a physical examination.
Next, the volunteer will undergo a venous assessment in the Department of Transfusion Medicine (DTM) on the day of screening. Approximately, 50 mL of venous blood will be required for screening. Baseline laboratory tests, including complete blood count (CBC) and differential, reticulocyte count, hemoglobin electrophoresis, type and screen, acute care panel, mineral panel, hepatic panel, markers of hemolysis (DAT, LDH, AST, ALT, and bilirubin), DTM viral screen, existence of pre-existing antibodies to biotin, urinalysis, and a complete medical history will be performed (Table 2). All patients who have received a transplant on a Gene Therapy protocol

may require additional confirmatory HIV testing which will also be performed under this protocol.

Participants with naturally occurring antibodies against BioRBCs will be excluded. All female volunteers of childbearing age will be required to undergo a serum pregnancy test. The screening visit will be used to determine initial eligibility for the protocol. Final subject eligibility will be confirmed once all lab results are noted in CRIS and eligibility criteria are met.

Once eligibility is confirmed, the participant will be scheduled for biotinylation of patient RBCs, and will have α-Globin genotype determined by Southern blot hybridization of genomic DNA extracted from peripheral blood leukocytes if the genotype is not already known.

Screen Failures: If a subject is found ineligible during the screening process and the subject will not meet the requirements within 30 days of the screening visit, the subject will be counted as a screen failure and removed from study.

The subject may re-screen if/when it is believed eligibility criteria may be met in accordance with the PI's discretion. Subjects will need to repeat the DTM viral screen during re-screening, as these lab results are no longer valid after 30 days. Any other test(s) or procedure(s) whose prior result rendered the subject ineligible needs to be repeated to see if more recent results would allow the subject to meet eligibility criteria. A new subject number will be assigned to a subject who is re-screened.

6.2 Biotinylation General: Collection of RBCs (as per section 6.3), biotinylation of patient RBCs, and re-infusion back to the patient within 48 hours as an outpatient. In the event the collection of RBCs occurs late in the day and biotinylation is unable to be performed the same day, RBCs will be stored overnight at 2-8°C. Biotinylation will be performed at room temperature under sterile conditions by the DTM. The goal total time during which the BioRBCs are at room temperature (i.e. during biotinylation and the transfusion procedure) should not exceed 4 hours. If immediate transfusion is not possible, the BioRBCs should be kept in a monitored refrigerator set at 4°C (range, 1-6°C).

6.3 Collection: On the day of blood collection, approximately 100 mL of venous blood will be collected via venipuncture for biotinylation of RBCs (~80 +/- 5mL), routine labs and alpha-Globin genotype (~20 mL). Alpha-Globin genotype will be determined by Southern blot hybridization of genomic DNA extracted from peripheral blood leukocytes if genotype is not already known. For participants without central venous access, an 18- or 20-gauge catheter will be placed in an adequate patient vein. Routine labs include approximately 20 mL of venous blood to provide a baseline complete blood count, reticulocyte count, DAT, and repeat pregnancy test (females only). In addition, whole blood is submitted for the purpose of DTM storage and will be held per DTM policies. DTM isolation procedures as defined in BB-DMF 11054 will be followed. The remaining approximately 70mL of whole blood will be collected in ACDA (Baxter healthcare Corp, Deerfield, IL) to obtain >15mL of packed RBCs (pRBCs). After verification that the DAT is negative, biotinylation of the collected sample may proceed.

6.4 Pre-Biotinylation Washing: The whole blood product will be visually inspected carefully; if clots/clumps are observed, product will be filtered before further processing. Blood will be centrifuged at 1000 x *g* (2142 rpm), deceleration (DEC) 4 at room temperature (RT; 17°C –25°C) for 10 minutes in table top centrifuge. The plasma layer will be aspirated and saved for use in reconstituting RBCs after biotin labeling. RBCs will be washed three times with 4 vol (4:1 wash:

RBCs) of PlasmaLyte A (Baxter healthcare Corp, Deerfield, IL). Washed RBCs will be resuspended in sufficient wash buffer to yield a 25% suspension of RBCs.

6.5 Preparation of Biotin: The CC-TAG-Biotin (ChemConnection BV, Netherlands) is stored in a -20ºC freezer and should be allowed to come to room temperature (~10-15min) before opening of the vial to avoid saturation of the biotin reagent. To prevent hydrolysis of the biotin reagent before addition to the RBCs, the biotinylation reagent will be prepared in wash buffer before use (within 20 minutes). A solution containing 1 mg/ml biotin will be prepared by dissolving 1 mg biotin in 1 mL PlasmaLyte A. This solution will then be sterilized by filtration through a 0.22-µm syringe filter (21062-25; Corning Glassware, Corning, NY).

6.6 Biotinylation Reaction: Biotinylation will be performed under a sterile Class 2 laminar flow hood in DTM. The appropriate volume of biotin solution will be added with gentle agitation to the 25% RBC suspension to give the desired final concentration of biotin of 18 µg/mL, incubated at room temperature for 30 minutes (Preclinical data shows efficiency of biotinylation of sickle RBCs from 1-80 µg/mL, however RBC morphology becomes deformed at concentrations >54 µg/mL. Flow cytometry peaks separating biotin labeled RBCs from unstained control is best at 18 µg/mL compared to lower biotin concentrations, without any evidence of RBC morphology changes at 18 µg/mL). The reaction will be terminated by sedimentation of RBCs at 1000 g (2142 rpm), deceleration (DEC) 4 at RT for 10 minutes followed by three 4:1 (vol/vol) buffer washes with PlasmaLyte A, sedimentation, and resuspension; this removes the remaining biotinylating reagent as well as any reaction and hydrolysis byproducts. The washed BioRBCs will be passed through an 18um filter (Hemo-Nate Syringe Filter, Utah Medical Products, Inc, Midvale, UT) and resuspended in autologous plasma at a hematocrit (Hct) of 50% (range 40-60%). 150 µL (range 50-500 µL) of pre-biotinylated RBCs and 150 µL of post-biotinylated RBCs will be withheld and sent to the Tisdale laboratory for pre-clinical studies (to ensure all cells are labeled and that there is a baseline separation of labeled and unlabeled cells on flow cytometric histogram) via methodology described below (see section 7.1).

6.7 Final Product: Final samples are removed for CBC, biotin labelling efficiency determination via flow cytometry, and safety assays (sterility and endotoxin). Release of the drug product is contingent on meeting the criteria listed in the table below (Table 1), which are also listed in the Certificate of Analysis in Module 3.2.P.5.4. For Safety testing, the final drug product must have an endotoxin level of ≤ 5 EU/mL and be negative for contamination in the sterility tests. Since the product is infused fresh, release for use is based on gram stain for sterility, with post-infusion testing based on a 14 day culture. The percent hematocrit and the percent of biotinylated RBC's will serve as a measure of purity and identity of the product.

Table 1: Drug Product Release Criteria

bioRBC Drug Product Release Testing	**Assay type**	**Method**	**Acceptable Limit**
Purity	**Appearance**	Visual check	Red; no clots or aggregates
Identity/Purity	**% HCT**	Advia CBC	50 ± 10%
Identity/Purity	**% Biotinylated RBC**	Flow	≥ 80%
Safety	**Endotoxin**	Limulus amebocyte lysate (**LAL**)	≤ 5 EU/mL

Safety	**Sterility**	Gram stain	No organisms seen
Safety	**Sterility***	Bacterial, BacT/ALERT bacteria culture*	No growth in 14 days*
Safety	**Sterility***	Fungal culture *	No growth in 14 days*

*Pending at release

6.8 Infusion of Product: For participants without central venous access, an 18- or 20-gauge catheter will be placed in an adequate patient vein for infusion of biotinylated RBCs (max volume 50 mL) and for obtaining immediate post-transfusion blood samples. Biotinylated RBCs will be reinfused per Nursing SOP for Cellular Therapy Products Administration; the time 20 minutes after the end of the infusion will be designated time zero (time for equilibration). The catheter will be flushed with 5mL normal saline at completion of the infusion. The time zero research lab can be drawn from the same IV in which biotinylated RBCs were infused.

6.9 Sample Collection: Study design, assessment parameters, and sample collection intervals (with approximate volume of blood required) are shown in Figure 1, Table 2, and Table 3, respectively. Approximately 5 mL of venous blood will be collected at 20 minutes after infusion for all participants, then approximately 10 mL will be collected at intervals as specified in Table 3 (+/- 3 days); non-transplant SCD participants will have samples collected bi-weekly for the first two weeks, then weekly until the percentage of biotinylated RBCs has decreased to the lower limit of detection (approximately less than 0.06%; estimate 1-8 weeks); all other participants (SCD post-transplant, sickle cell trait, healthy volunteers) will have samples drawn weekly for the first 4 weeks (starting week 2 through week 4) then every other week until the percentage of biotinylated RBCs has decreased to the lower limit of detection (approximately less than 0.06%; estimate 12-16 weeks) (Table 3). All participants will return for anti-biotin antibody detection at 6 months post-infusion (end of study). Samples will be analyzed as described below (sections 7.1-7.5) for % survival biotinylated RBCs, hemoglobin content, F cell percentage, and delay time analysis, with the remainder of the sample sent to the clinical lab, DTM, and the Tisdale lab for determination of Hb, Hct, reticulocyte count, LDH, AST, ALT, bilirubin, DAT, and detection of antibodies to biotinylated RBCs (see section 6.10)(Table 2). At every encounter, participants will be asked about any illness since their last visit.

Table 2 Assessment Parameters

Visit	Parameter	Additional History
Screening	CBC Reticulocyte count Hemoglobin electrophoresis Acute Care Panel Mineral Panel Hepatic Panel DAT ABO/Rh and antibody screen LDH Biotin antibody screen* Serum pregnancy test (females) **HIV-1/HCV/HBV (NAT) Hepatitis B (surface antigen) Hepatitis B (core antibody) Hepatitis C (antibody)	Complete history including past medical history, transfusion history, bleeding history, and medications. A complete SCD history (where applicable) will include hydroxyurea use and dose, pain crisis frequency, ER and hospitalization utilization, and transfusion history.

	HTLV I and II (antibody) **HIV 1 and 2 (antibody) West Nile Virus T cruzi (antibody) Treponema pallidum (antibody) or equivalent (such as RPR) Urinalysis		
Week 1/Day -1 (Blood Collection)	CBC Reticulocyte count DAT Serum pregnancy test (females) α-Globin genotype Infectious screening of whole blood per DTM (see 6.3)		Interim illness update (specifically for SCD patients, history of pain crisis, transfusion, ER visit, or recent hospitalization)
Week 1, Day 1 (Infusion Day)	*For Tisdale Lab& bluebird bio*		
	% survival biotinylated RBCs Hemoglobin content F cell percentage Delay time analysis HbF staining HbS single cell Western Blot Assay development		
Study Assessments (Time Zero – End of Study*)	*Clinical Lab & DTM*	*Tisdale & Eaton Labs, bluebird bio*	
	CBC Reticulocyte count Acute Care Panel Mineral Panel Hepatic Panel LDH DAT ABO/Rh and antibody screen Hemoglobin electrophoresis Biotin Antibody Detection*	% survival biotinylated RBCs Hemoglobin content F cell percentage Delay time analysis HbF staining HbS single cell Western Blot Assay development	Interim illness update as above

*Biotin antibody detection will be done at initial screen, during the course of the study if bioRBC degradation is faster than predicted, and at 3 and 6 months post-infusion for all patients regardless of end of study date.

**Gene Therapy patients may have other HIV testing, including HIV ½ Antibody/Ag Combo.

Table 3. Sample Collection Intervals

		Week 1/Day 0, Infusion Day		Week^																
	Screening	Pre-infusion	Post-infusion	1	2	2*	3	4	5	6	7	8	9	10	11	12	13	14	15	16	24
SCD Pre-Transplantation	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
SCD Post-Transplantation	X	X	X		X		X	X		X		X		X		X		X		X	X
HbAS	X	X	X		X		X	X		X		X		X		X		X		X	X
HbAA	X	X	X		X		X	X		X		X		X		X		X		X	X
Approximate Volume of Blood (mL)	50	100	5	**10 (per sample collected)**																	

^All patients will follow the sample interval until the percentage of biotinylated RBCs has decreased to the lower limit of detection (approximately <0.06%), at which time the next interval for that patient will be the final 6 month (week 24) interval.
2*= Bi-week 2

6.10 Antibody Detection: To test for antibodies to biotinylated RBCs, plasma samples will be tested using gel card antibody detection(MTS anti-IgG Card, MTS084024, Ortho Clinical Diagnostics) as previously described (11). The detection of specific antibodies to bioRBC does not determine whether these antibodies are physiologically active or are clinically significant. Thus far, all identified pre-existing, naturally occurring anti-bioRBC antibodies are neutralized by biotin compounds, while induced anti-bioRBC antibodies are expected to be inhibited by bioRBC and not by free biotin as previously published (11). It is speculated that this is analogous to penicillin-type drug-dependent antibodies with biotin covalently attached to RBC surface proteins acting as immunogenicity enhancing haptens. In those reported cases of anti-bioRBC antibody detection, biotinylation occurred at higher concentrations (54-162 µg/mL), antibodies were transient and no longer detected after 6 months, and did not alter the red cell survival (4,11).

7.0 Analysis of the Study

7.1 Flow Cytometry Analysis: Enumeration of BioRBCs will be performed by flow cytometry. Samples will be collected pre and post biotinylation prior to infusion (as per section 6.6), at 20 minutes post infusion (time zero), and as described in section 6.9 & Figure 1. To isolate the RBC fraction, 40 uL of whole blood aliquoted in triplicate will be mixed with 1 mL of wash buffer, centrifuged at 10,000 x *g* until the rpm value is reached, and supernatant will be discarded (repeat x1). The final washed RBC pellet will be resuspended in 80 µL of wash buffer, mixed with 20 µl of a 1:20 dilution (in PBS) of streptavidin-phycoerythrin (SA-PE; S-866; Molecular Probes, Eugene, OR), and incubated for 30 min at room temperature in the dark. The staining reaction is stopped by adding 3 mL of wash buffer, centrifuging at 1500 rpm for 5 minutes, and removing the aspirate. RBCs will then be suspended in 500 µL of wash buffer, and will be filtered through a 0.45 µm filter prior to flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). Percentages of BioRBCs will then be measured by flow cytometry.

7.2 F-cell and S-cell measurement: Simultaneously with the measurement of BioRBCs by flow cytometry (see section 7.1), samples will be analyzed with an additional color (3-color flow cytometry) for Biotin, HbF, and HbS. After incubation with SA-PE and two washes, the cells will be mixed with a 2µL/mL solution of glutaraldehyde/PBS. Cells will be gently mixed and incubated at room temperature for 10 minutes. Cells will be centrifuged at 2500 rpm x 5 min, then incubated at room temperature with 1mL 0.1% Triton X solution for 10 minutes. After centrifugation at 2500 rpm x 5 minutes, cells will be stained for 30 minutes at room temperature with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to HbS and allophycocyanin (APC)-conjugated monoclonal antibody to HbFprior to analysis by flow

cytometry. The F per F cell can be calculated from the percent F cells, the percent HbF (determined by HPLC, see section 7.3), and the MCH.

7.3 Hb Content: BioRBCs will be isolated from post-infusion blood samples using streptavidin-coated magnetic beads or flow cytometry separation. The collected BioRBCs and the negative fraction will be analyzed by high performance liquid chromatography (HPLC) for hemoglobin content.

7.4 Measurements of delay time for sickling of individual red cells: Blood from patients will be diluted 1500 to 3000-fold, depending on the hematocrit, into PBS. 10 µL of the cell suspension is added to the wells of a 384-well plate. The well plate is inserted into a humidified chamber of a Biotek "Lionheart FX Automated Microscope System" and, for patients with SS disease, deoxygenated with a mixture of 96% nitrogen and 4% oxygen to induce sickling. Everything that follows is fully automated. Images taken in 430 nm light (the absorption peak of deoxyhemoglobin) of 100-200 cells settled on the bottom of each of the 384 wells are measured every 15 minutes for up to 8 hours (see Figure 2). A single scan of all 384 wells takes 15 minutes because the autofocusing system of the instrument requires about 2.3 seconds to obtain a focused image from each well and 56 seconds to scan a row of 24 wells. Because images are taken of 40,000 to 80,000 cells, excellent statistics can be obtained on more than 20 different blood samples in a single experiment (using less than about 100 nanoliters from each patient). Sickling times are determined with our in-house developed, robust image analysis software (12), which compares several metrics of the cell image to determine in which 15 minute interval the cell has become distorted, i.e. sickled. These include the eccentricity (deviation from a circular projection) and ratio of the optical density at the periphery of the cell to that at the cell center (unsickled cell is more transparent in the center). From the fraction sickle vs time (Figure 3), the final output for each blood sample is a t50 for sickling. The comparison between SS and AS cells shows the enormous sensitivity to the intracellular hemoglobin composition (> 90% Hb S in SS cells; 60/40 HbA/Hb S mixture in AS cells).

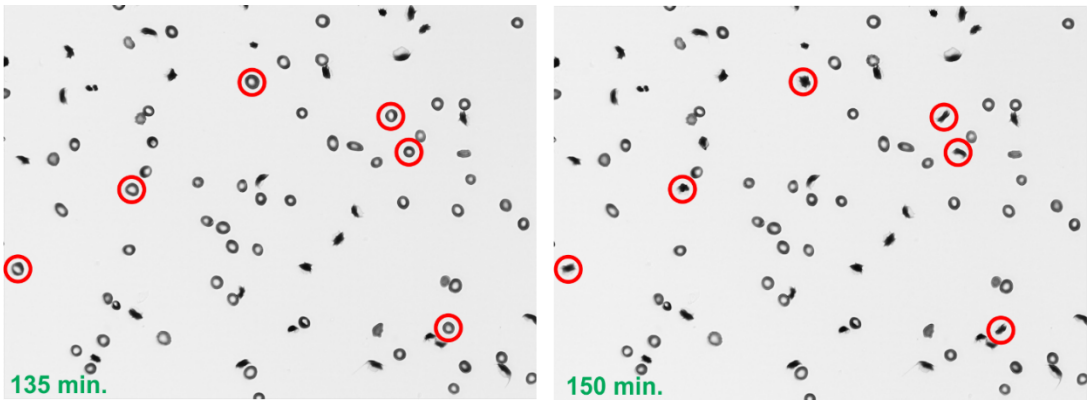

Figure 2. Images of cells at 135 and 150 minutes after the start of deoxygenation with 96% nitrogen and 4% oxygen. Cells that sickle between 135 and 150 minutes are circled. At 150 minutes 37 % of the 84 cells are sickled.

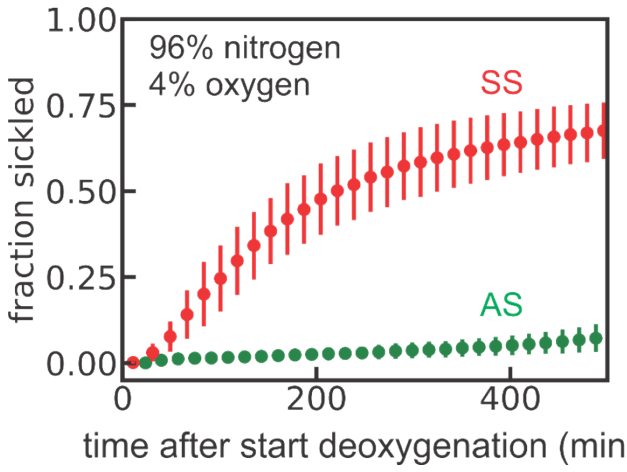

Figure 3. Mean fraction sickled as a function of time following deoxygenation of SS and AS cells with 96% nitrogen and 4% oxygen. The error bars correspond to one standard deviation from the mean of the fraction sickled in 96 wells. They are about twice the expected value due to the finite number (~100) of cells in each well. The higher standard deviations compared to AS is mainly caused by small differences in the geometry of each of the 10 microliter droplets, which result in differences in the fraction sickled at the same deoxygenation time due to differences in the rate of oxygen diffusion out of the droplet. The fraction sickled vs deoxygenation time for the SS cells is very similar to the kinetics for AS cells when deoxygenated with 100% nitrogen.

7.5 Other Analysis: A portion of collected RBCs will be used for imaging flow cytometry, HbF staining, HbS single cell Western Blot, and assay development. Duplicate samples of approximately 2 mL (range 1-5 mL) of pre-infusion, time zero, and subsequent patient samples will be sent to our collaborators at bluebird bio Inc, for parallel analysis and assay development. No samples will be sent to our collaborators until a Material Transfer Agreement (MTA) has been established.

Another portion of collected RBCs will be used for measurement of glycolytic metabolites. Duplicated coded samples of approximately 450 uL will be sent to our collaborators at Agios Pharmaceuticals, Inc. for parallel analysis. The results of these findings will be reidentified at the NIH to answer this new analysis. An MTA for this collaboration has been fully executed.

7.6 Data and Statistical Analysis: This is an exploratory study designed to test methodology and assay development for the purposes of studying red cell survival in patients with SCD. RBC survival curves will be generated for each participant. Mean potential RBC life span (MPL) will be determined by plotting the percentage of remaining biotinylated RBCs in circulation against time after transfusion. Median RBC survival and mean RBC age will be calculated from these curves. Correlation of mean RBC survival with known markers of red cell survival will be performed using Pearson Correlation. The mean of each participant's clinical laboratory values during the study period will be used for correlation with RBC survival. Average MPL among the groups studied will be examined descriptively using means and standard deviations. The differences between groups will be used to examine the feasibility and guide the design of larger, definitive studies; in this pilot study, statistically significant differences are not generally anticipated. Similarly, information regarding hydroxyurea use, α globin genotype, etc are likely to not be powered to see an effect.

7.7 Mathematical Model: We assume that in erythropoietic homeostasis, RBC lifetime is proportional to the inverse of the reticulocyte fraction such that RBC survival is lowest in patients with the highest reticulocyte counts. We will subsequently develop a mathematical model to predict the percentage of necessary hemoglobin to reverse SCD from reticulocyte count and RBC half-life.

7.8 Off Study Criteria: Patients reserve the right to come off study for any reason during the study period. Patients may be taken off-study by the PI if there is a health-related event during the course of the study that affects RBCs lifespan or production of RBCs including hemorrhage or need for transfusion. In the event of pregnancy after infusion of biotinylated RBCs, the patient will remain on study unless directed otherwise by the patient.

8.0 Drug Formulation, Availability, and Preparation

- Biotin

  CC TAG BIOTIN ® (Biotin 3 sulfo N Hydroxysuccinimide ester sodium salt) is derived from the natural form of biotin, which is the D enantiomer (Vitamin B7 or B8 according to nomenclature of each country). Because of its small molecular size CC TAG Biotin (small spacer arm: 13.5 Å, between the biotin and the sulfo NHS group) can be conjugated to many proteins without altering their biological activities.

  Sulfo NHS activated biotin reacts efficiently with primary amino groups (-NH2) in pH 7-9 buffers to form stable and covalent amide bonds. Proteins from plasma membrane cells generally have several primary amines in the side chain of lysine (K) residues that are available as targets for attachment of biotin. Biotin binds with high affinity to avidin and streptavidin proteins. Therefore the tracking of biotin labelled cells with the CC TAG BIOTIN ® can be performed easily by flow cytometry with fluorescent streptavidin. Biotin labelled proteins can also be easily detected in ELISA, dot blot or Western blot applications using streptavidin or avidin conjugated probes.

  The new cGMP CC-TAG-BIOTIN® has been demonstrated to be efficient in labeling blood cells (RBC, WBC and platelets) *ex vivo*. It will reduce the safety concerns and contribute to overcome the need for non- radioactive methods within the framework of *in vivo* studies in humans.

  Supply: ChemConnection. (commercially available)

  Preparation: As per section 6.5, CC-TAG-Biotin is stored in a -20ºC freezer and should be allowed to come to room temperature (~10-15min) before opening of the vial to avoid saturation of the biotin reagent. To prevent hydrolysis of the biotin reagent before addition to the RBCs, the biotinylation reagent will be prepared in wash buffer before use (within 20 minutes). A solution containing 1 mg/ml biotin will be prepared by dissolving 1 mg biotin in 1 mL PlasmaLyte A. This solution will then be sterilized by filtration through a 0.22-µm syringe filter (21062-25; Corning Glassware, Corning, NY).

  Storage: CC-TAG-Biotin is moisture-sensitive. Store reagent at -20°C in the original container with desiccant. To avoid moisture condensation onto the product, vial must be equilibrated to room temperature before opening.

  Route of Administration: As per section 6.6 and 6.8, biotin will be applied ex vivo to patient RBCs, then biotinylated RBCs will be reinfused intravenously to the patient.

9.0 Data and Safety Monitoring Plan

The side effects of blood donation and transfusion are well described and vast experience with the procedure exists in the Clinical Center.

Principal Investigator: Accrual and safety data will be monitored by the Principal Investigator, Dr. John Tisdale, who will provide oversight for the conduct of this study. The protocol will be

continuously evaluated for any unusual or unpredicted complications with the aim of detecting and preventing unacceptable increase in morbidity and mortality over and above that anticipated from sickle cell disease or other hemoglobinopathies.

NIH IRB: Accrual and safety data will be monitored and reviewed annually by the Institutional Review Board (IRB). Prior to implementation of this study, the protocol and the proposed patient consent and assent forms will be reviewed and approved by the properly constituted Institutional Review Board (IRB) operating according to the 45 CFR 46 Code of Federal regulations. This committee must approve all amendments to the protocol or informed consent, and conduct continuing annual review so long as the protocol is open to accrual or follow up of subjects. Quality assurance and control monitoring will be consistent with the NHLBI Division of Intramural Research Clinical Research Quality Assurance and Quality Control Policy.

10.0 Adverse Events, Deviations, and Unanticipated Problem Assessments

10.1 Definitions

Please refer to definitions provided in Policy 801: Reporting Research Events.

10.2 Adverse Event Assessment

The following adverse event management guidelines are intended to ensure the safety of each participant while on the study. The AEs will be attributed (unrelated, unlikely, possibly, probably or definitely) to study procedure and/or disease and graded by severity utilizing CTCAE version 5.0. A copy of the criteria can be downloaded from the CTEP home page at http://ctep.cancer.gov/reporting/ctc.html.

Biotin labeling of RBCs is not expected to result in adverse events. However, all Grade 2 and above adverse events (AE) that are related to diagnostic and interventions directly related to this study whether volunteered by the participant, discovered by study personnel during questioning, or detected through physical examination, clinically significant laboratory test, or other means will be recorded in the participant's medical record.

Only Grade 2 and above adverse events (AE) that are related to diagnostic and interventions directly related to this study will be captured in the research database. Adverse events associated with the natural history of sickle cell disease will not be captured in the database. Expected sequelae of sickle cell disease such as vaso-occlusive pain crisis, acute chest syndrome or cytopenias associated with hydroxyurea therapy will not be captured in the database.

10. 3 NIH Intramural and IRB Reporting

Expedited Reporting
Events requiring expedited reporting will be submitted to the IRB per Policy 801 "Reporting Research Events".

Reports to the IRB at the time of Continuing Review:
The PI or designee will refer to HRPP Policy 801 "Reporting Research Events" to determine IRB reporting requirements.

Reports to the CD:
The PI or designee will refer to NHLBI DIR guidelines to determine CD reporting requirements and timelines.

10.4 Protocol Deviations

It is the responsibility of the investigator to use continuous vigilance to identify and report deviations to the NIH Institutional Review Board as per Policy 801.

NIH Definition

A protocol deviation is any changed, divergence, or departure from the IRB-approved research protocol.

- Major deviations: Deviations from the IRB approved protocol that have, or may have the potential to, negatively impact the rights, welfare or safety of the participant, or to substantially negatively impact the scientific integrity or validity of the study.
- Minor deviations: Deviations that do not have the potential to negatively impact the rights, safety or welfare of participants or others, or the scientific integrity or validity of the study.

10.5 Unanticipated Problems

The investigator will report unanticipated problems (UPs) to the NIH Institutional Review Board (IRB) as per Policy 801.

Definition:

Any incident, experience, or outcome that meets all of the following criteria:

- Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol-related documents, such as the Institutional Review Board (IRB)-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied; and
- Related or possibly related to participation in the research ("possibly related" means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- Suggests that the research places participants or others (which many include research staff, family members or other individuals not directly participating in the research) at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or expected.

Reporting:

The investigator will report unanticipated problems (UPs) to the NIH Institutional Review Board (IRB) as per Policy 801 and to the NHLBI Clinical Director per NHLBI guidelines.

11.0 Specimen and Data Storage, Tracking and Disposition

11.1 Intended Use of the Samples/Specimens/Data

Biospecimen Management

Specimens and their derivatives (e.g., genomic material, cell lines) will be coded and stored in conformity with DIR Policy. Coded biospecimens may be sent to collaborators outside of the NIH with IRB approval in accordance with applicable NIH and DIR Policy for sharing research resources, including an executed material transfer agreement. Biospecimens with participant personal identifiers may be sent to associate investigators and collaborators outside of the NIH only after approvals of both NHLBI and local IRBs, an executed reliance agreement with NHLBI's IRB, or an extension of the NIH's FWA through an Individual Investigator Agreement.

Any future research use of biospecimens not defined in the protocol in which NHLBI investigators are engaged in research (e.g., they are undertaking research activities and hold the key that identifies research subjects) requires IRB review and approval. Coded biospecimens (NHLBI investigators hold the key that identifies research subjects) to be shared outside of NIH for future research use where results will not be returned to the Principal Investigator does not require IRB review or approval.

11.2 How Samples will be Stored

Data Management

The PI will be responsible for overseeing entry of these data into an in-house password protected electronic system at the NIH and ensuring data accuracy, consistency, and timeliness. The principal investigator and associate investigators, research nurses and/or a contracted data manager will assist with the data management efforts. Data will be abstracted from Clinical Center progress notes as well as reports in CRIS from tests performed at the Clinical Center. Laboratory data from NIH will be reviewed using CRIS or an approved NHLBI database.

All human subjects' personally identifiable information (PII), eligibility and consent verification will be recorded. Primary data obtained during the conduct of the protocol will be kept in secure network drives or in approved alternative sites that comply with NIH security standards. Primary and final analyzed data will have identifiers so that research data can be attributed to an individual human subject participant, (e.g., a unique code or minimum PII required for subject identification). Genetic information will not be shared with anyone besides the protocol analysis team, and these research data will be maintained on a secure server at NIH.

Sample Storage

Access to research samples will be limited. The samples will be stored in the secure laboratory of the principal investigator and placed in locked freezers. Only those authorized can retrieve samples. Samples and data will be stored using codes assigned by the investigators or their designee(s). Only investigators or their designee(s) will have access to the samples and data. The PI will be responsible for overseeing entry of data into an in-house password protected electronic system. Research samples will be stored in accordance with NHLBI DIR Biospecimen policy.

11.3 How Specimens/Data will be Tracked

Samples collected at the NIH will be ordered and tracked through CRIS Research Screens. Should a CRIS screen not be available, the NIH form 2803-1 will be completed and will accompany the specimen and be filed in the medical record.

All samples collected at the NIH under this protocol will be coded with a specific patient number (i.e. 161-1, 161-2, etc.). They will be logged in on an excel spreadsheet containing the sample code, date drawn, and location of storage.

All medical information collected from study participants at the NIH will be kept in a locked file at the Clinical Center at the NIH. Unique patient identifiers will be used to label all data. Strict standards of confidentiality will be upheld at all times.

11.4. Publication, Data Sharing/Future Use of Data

De-identified human data generated for use in future and ongoing research will be shared through a NIH-funded or approved repository (ClinicalTrials.gov) and BTRIS. At the completion of data

analysis, data will be submitted to ClinicalTrials.gov either before publication or at the time of publication or shortly thereafter.

Research data may be shared with qualified non-collaborator recipients following publication of the primary research results after removal of PII. Future research use of identifiable data not defined in the research protocol may occur only after IRB review and approval. Refusal of a research subject participant to permit future use of data will be honored.

Blood samples may be used for other research purposes related to the study of SCD. Volunteer clinical data will be protected and tracked using standard operating procedures in the medical record department. No volunteer data will be stored in the research office.

Following collection and processing, identifiers will be stripped from the samples prior to their distribution to other study investigators. The IRB will be notified if research samples are unintentionally destroyed or lost.

Transmission of Data to Outside Investigators: Coded samples and data will be sent to our collaborators for analysis. No samples or data collected on this study will be sent outside NIH without a fully executed material transfer agreement (MTA).

11.5 End of Study Procedures

The study will remain open so long as sample or data analysis continue. Samples from consenting participants will be stored until they are no longer of scientific value or if a participant withdraws consent for their continued use, at which time they will be destroyed. A file note will be made to document the withdrawal / destruction, and existing data will not be used in future studies.
At the completion of the protocol (termination), samples and data will either be destroyed, or after IRB approval, transferred to another existing protocol or a repository.

11.6 Loss or Destruction of Specimens/Data

Any loss or unanticipated destruction of samples (for example, due to freezer malfunction) or data (for example, misplacing a printout of data with identifiers) will be reported to the IRB. Additionally, participants may decide at any point not to have their samples stored. In this case, the principal investigator will destroy all known remaining samples and report what was done to both the participant and to the IRB. This decision may not affect the participant's participation in this protocol or any other protocols at NIH.

12.0 Confidentiality and Privacy

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and the sponsor(s) and their interventions. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor.

All research activities will be conducted in as private a setting as possible.

The study monitor, other authorized representatives of the sponsor, representatives of the Institutional Review Board (IRB), and/or regulatory agencies may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB, Institutional policies, or sponsor requirements.

To further protect the privacy of study participants, a Certificate of Confidentiality has been issued by the National Institutes of Health (NIH). This certificate protects identifiable research information from forced disclosure. It allows the investigator and others who have access to research records to refuse to disclose identifying information on research participation in any civil, criminal, administrative, legislative, or other proceeding, whether at the federal, state, or local level. By protecting researchers and institutions from being compelled to disclose information that would identify research participants, Certificates of Confidentiality help achieve the research objectives and promote participation in studies by helping assure confidentiality and privacy to participants.

13.0 Human Subjects Protections

13.1 Rationale for Exclusion of Children

This is a non-treatment study with no potential for direct benefit to the subject.

13.2 Rationale for Exclusion of Subjects Unable to Consent

This is a non-treatment study with no potential for direct benefit to the subject.

13.3 Rationale for Exclusion of Pregnant Women

This is a non-treatment study with no potential for direct benefit to the subject. Furthermore, the natural increase in circulating blood volume associated with pregnancy may render the small volume of biotinylated RBCs described in this study unable to be detected by analysis.

13.4 Rationale for Exclusion of Subjects with Pre-existing Antibodies to Biotin

This is a non-treatment study with no potential for direct benefit to the subject. Though the existence of antibodies to BioRBCs has not demonstrated a shortened RBC lifespan (4, 11), data is limited in subjects with sickle cell disease.

13.5 Inclusion of NIH Staff

NIH staff (employees, NIH contractors, special volunteers, guest researchers, and trainees) may voluntarily participate in this protocol.

• If the individual requesting to participate in the protocol is a co-worker, the consent from the NIH staff member (co-worker) will not be obtained by the staff member's direct supervisor but by another research staff member approved for obtaining informed consent who is not a co-worker.

• Recruitment, enrollment and compensation of NIH employee subjects will be consistent with the Guidelines for the Inclusion of Staff in NIH Intramural Research Studies (NIH HRPP Policy 404) and NIH Policy Manual Chapter 2300-630-3 and the Leave Policy for NIH Employees Participating in NIH Medical Research Studies.

• Neither participation nor refusal to participate as a subject in this protocol will have an effect, either beneficial or adverse, on the participant's employment or position at NIH.

Known Potential Risks

13.6 Known Potential Risks

Blood Draw: A potential risk of participation in the protocol involves the risk associated with drawing blood. The potential complications of phlebotomy involve minimal risk and consist of pain, mild bruising at the site of venipuncture and potential local infection.

HIV Testing: Testing for infection with the human immunodeficiency virus (HIV) may be performed more than once for this study. If the subject is infected with HIV, they will not be able to participate in this study. We will inform the subject of the results, what they mean, how to find care, how to avoid infecting others, how we report HIV infection, and the importance of informing their partners at possible risk because of the subject's HIV infection.

Risks related to protection of confidentiality: The information from this study will be maintained confidential. Samples will be assigned a unique code prior to testing that will serve as a link to the individual's identity and other information collected as part of this research protocol. The actual testing will be performed on coded samples.

Any breach of the secure database or accidental release of a code key for linked samples will be reported to the IRB immediately as an adverse event. All efforts will be made to protect confidentiality of subjects to the extent possible by law.

Risks related to Biotin

Background:

Biotin is a water-soluble B-vitamin, also called vitamin B7. Biotinylation is the rapid and specific process of covalently attaching biotin to a protein, nucleic acid, or other macromolecule, and is unlikely to perturb the natural function of the molecule due to the small size of biotin (MW = 244.31 g/mol) (13). Biotin forms a covalent amido bond between the carboxyl group of biotin and the epsilon amino group of lysine residues in the RBC membrane proteins (Figure 4). Biotin binds to streptavidin and avidin with an extremely high affinity, fast on-rate, and high specificity, and is a useful tool in nonradioactive methods of purification, detection, immobilization, labeling, viral vector-targeting, and drug targeting systems. Proteins that are biotin labeled are routinely detected or purified with avidin conjugates in many protein research applications, including cell surface labeling and flow cytometry/fluorescence-activated cell sorting.

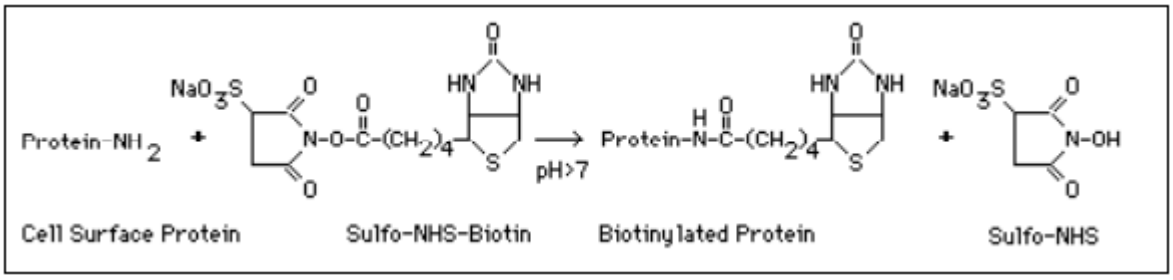

Figure 4 Biotin Structure. The reaction of sulfo-NHS biotin targets the amino groups on RBC surface proteins.

Expected Side Effects:

Previous studies with identical or similar protocols have shown the safety of biotinylated RBCs in human subjects for determining red cell survival (4, 14-15). As these groups have surmised, because the biotin reaction with the amino group produces the same biotinylation of the epsilon amino group of lysine seen in biotin-dependent carboxylases and biotinylated histones and is not known to generate toxic byproducts, we speculate that no toxicity will be observed with the use of biotinylated RBC in human subjects.

Furthermore, biotin itself will not be administered to subjects, nor will the biotinylating reagent. Only RBCs after they have been biotinylated using the sulfo-NHS biotinylating reagent and washed (thus eliminating the excess biotinylating reagent) will be used.

Common Side Effects:
We do not expect side effects directly from the use of biotin. Adverse reactions related to blood draws may occur, include lightheadedness, nausea, and pre-syncope due to vasovagal reactions. Vasovagal reactions are managed by postural manipulation and volume administration. Further, pain and bruising can result from needle sticks required for blood draws.

Rare Side Effects:
Many lab tests use biotin technology due to its ability to bond with specific proteins which can then be measured to detect certain health conditions. Patients who are ingesting high levels of biotin in dietary supplements (ingested at 650 times the recommended daily intake promoted for hair, skin, and nail benefits, or recommended for patients with certain conditions such as multiple sclerosis) can cause clinically significant incorrect lab test results (16). Biotin levels higher than the recommended daily allowance (RDA, 0.03mg) may cause interference with lab tests. The amount of biotin used in this study is well below the RDA, therefore is not expected to interfere with any clinical laboratory tests.

A theoretical safety issue is the development of anti-BioRBCs after BioRBC exposure. Previous studies have detected transient BioRBC-induced anti-BioRBC, however these antibodies have not caused clinical abnormalities (4,11). It is speculated that this is analogous to penicillin-type drug-dependent antibodies with biotin covalently attached to RBC surface proteins acting as immunogenicity enhancing haptens. Clinical features of hemolytic reactions have not been seen—presumably because the volumes of BioRBCs transfused for RBC kinetic studies are small (approx. 0.40 mL of BioRBC/kg body weight) and unlikely to cause a clinically significant transfusion reaction, even if the entire BioRBC transfusion was acutely hemolyzed (11).

13.7 Known Potential Benefits
This is a non-treatment study with no potential for direct benefit to the subject. The collection and biotinylation of RBCs in this study will allow the measurement and study of red cell survival in patients with and without SCD. The study of RBC survival and its relation to known influencers of red cell survival including ARC, % HbF, and F cell percentage will allow for the creation of a mathematical model that will help determine necessary amount of normal hemoglobin required to reverse the SCD phenotype. Such determination has the potential to influence current and future gene therapy protocols. With the success of this pilot study, this methodology can be expanded to include the ability to measure RBC lifespan in gene therapy patients, and the relationship of transferred globin (VCN) and percent engraftment of gene modified cells to the patient's RBC survival. As has been previously described (10), the reticulocyte fraction is a good proxy for RBC lifetime, and therefore gene therapy may be most efficacious in patients in which RBC survival is lowest (with highest reticulocyte count) as they would require lower amounts of normal hemoglobin production (lower levels of gene correction) to sufficiently reverse the SCD phenotype. A study of red cell survival will aid in this determination of the amount of hemoglobin transfer necessary for any particular individual with SCD.

13.8 Assessment of Potential Risks and Benefits
Risks involved in participation in this study are low and include risks related to blood draws, risks related to protection of confidentiality, and risks related to biotin (see section 12.6). We do not expect to see side effects related to biotinylation of RBCs, however the theoretical safety issue of development of anti-BioRBCs after BioRBC exposure is addressed by screening before, during, and after completion of the study for anti-BioRBCs. Previous studies that have detected BioRBC-induced anti-BioRBC describe

these antibodies as transient with no clinical changes presumably because the volumes of BioRBCs transfused for RBC kinetic studies are small (approximately 0.40 mL of BioRBC/kg body weight) and unlikely to cause a clinically significant transfusion reaction, even if the entire BioRBC transfusion was acutely hemolyzed (4,11). The knowledge gained from this study including RBC survival in patients with SCD before and after BMT, and RBC survival in subjects with SCT (not previously done with biotinylation), will provide essential information that will inform future gene therapy studies while exposing participants to minimal risk.

13.8 Informed Consent Processes and Procedures

Informed consent will be conducted following *OHSRP Policy 301- Informed Consent*.

An IRB-approved consent form will be provided to the subject electronically or by hard copy for review prior to consenting. The investigational nature and objectives of this trial, the procedures, and their attendant risks and discomforts and potential benefits will be carefully explained to the subject in a private setting. The subject will be given as much time as they need to review the document and to consult with their family, friends, and personal health care providers. In addition, a study team member will be available to answer any questions.

A signed and dated informed consent document will be obtained by any investigator authorized to consent prior to entry onto the study. Consent may be obtained with required signatures on the hard copy of the consent or on the electronic document.

When a document that is in electronic format is used for obtaining consent, this study may use the iMed platform which is 21 CFR, Part 11 compliant, to obtain the required signatures.

During the consent process, participants and investigators may view the same approved consent document simultaneously when participant is being consented in person at the Clinical Center or both may view individual copies of the approved consent document on screens in their respective locations remotely. Signatures may be obtained either by both directly signing on the device that the consenting investigator is using (when in person) or through iMed Mobile Signature Capture (remotely) which allows texting or emailing a link to the participant. That link allows the participant to review the consent, then proceed to sign on the device they are using.

Whether hard copy or electronic consent, both the investigator and the participant will sign the document with a hand signature using a pen, finger, stylus, or mouse.

When done remotely, if the participant prefers to sign a hard copy, they may be instructed to sign and date the consent document during the discussion and mail, secure email or fax the signed document to the consenting investigator.

Whether in person or remotely, the privacy of the participant will be maintained.

Finally, the fully signed informed consent document will be stored in the electronic medical record, and the subject will receive a copy of the signed informed consent document.

13.9 Patient Advocate

A patient's rights representative is available to patients on this protocol. The representative is located in Building 10 and can be reached by phone at (301) 496-2626. Patients may ask any questions about the study and may withdraw their consent at any time.

14.0 Compensation for Participation

Clinical research volunteers evaluated at the Clinical Research Center will receive monetary compensation in accordance with NIH guidelines. NIH employees may take part in this study as volunteers and receive compensation, as outlined below, in accordance with the NIH leave policy. Patients with a disease may be compensated as volunteers if testing is done solely for use as controls to study other disease processes.

Outpatients (on-site): **$20 for first hour or part thereof and $10 each additional hour or part thereof (up to 4 hours).**

Procedure	**Inconvenience units ($10 for each inconvenience unit)**
Outpatient Visit (first hour)	$20 (2 units)
Outpatient Visit (additional hours, up to 4 hours)	$10 (1 unit)
Screening History & Physical	$25 (2.5 units)**
Research blood sampling (per blood draw)	$40 (4 units)
IV Placement	$10 (1 unit)
Drug Infusion/Administration by IV	$50 (5 units)
Completion Bonus	$500 (50 units)
Travel Voucher (per visit)	$40 (4 units)
Total Potential Compensation	Up to $1,600.00*

*Dependent on the subject's red cell half- life, the number of research samples needed to collect until study completion, although rare, may exceed the projected 16 week collection timeline. Therefore total compensation may exceed $1,600.00.
**Unless screened under another NIH protocol.

15.0 Reimbursement for Travel

Reimbursement will be consistent with NHLBI DIR Travel and Lodging Compensation of Clinical Research Subjects policy. Please reference the table above in Section 13.0 *Compensation for Participation,* this study offers a $40 voucher for travel per visit; however, it does not offer reimbursement for, or payment for lodging and/or meals.

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APPENDIX A: NIH INFORMATION SHEET ON STAFF RESEARCH PARTICIPATION (April 2016)

As an NIH employee, contractor, Special Volunteer, Guest Researcher, or trainee, you may participate in intramural research studies unless it is prohibited by your Institute or Center (IC), or if you are excluded by the criteria of the protocol in which you want to enroll. The inclusion of NIH staff in a particular protocol must also be approved by the IRB. You may be motivated by altruism, a commitment to research in your own or related fields, or want access to clinical trials of potential direct therapeutic benefit. When deciding, you should make an informed decision about participation. This information sheet offers some points to consider for NIH staff who are considering research participation at NIH.

First, similar to any individual who is considering research participation, you should seek adequate information about the study purpose, what is required of you in terms of procedures, interventions and time, and the potential risks and benefits of participation. For more information, see the NIH Clinical Center's public website "Are Clinical Studies for You?" at: http://www.cc.nih.gov/participate/studies.shtml.

When you are thinking about participation in a research study that is being conducted by your supervisor, or others with whom you work closely in your laboratory, branch, or unit, you should consider some additional factors:

A. Possible bias: Are you confident that you can be unbiased about reporting answers, side effects, or other information that could influence the study outcome or risk to you?

B. Confidentiality: Has the principal investigator (PI) spoken about what information will be collected from you as part of the study? Has the PI discussed what information will be available to those within, and outside, the study team? If applicable, are you comfortable sharing your medical history (including, for example, mental health history or STDs) and your social history (e.g. substance use) with study investigators who may be your coworkers, or with the possibility of them discovering something about your health during the study (e.g. pregnancy status or a new diagnosis)? Although every effort will be made to protect your information and keep it private and confidential, your information may, depending on the nature of the protocol, become available in medical records or to authorized users outside of the study team. Discuss any concerns with the PI.

C. Pressure: Do you perceive any pressure or expectations from your supervisor or colleagues regarding participation? Could that pressure influence your decision or make it difficult for you to choose whether or not to participate? Remember that it is your choice whether or not to participate and that your decision to participate or not should not have an effect, either beneficial or adverse, on your position at NIH.

D. Time and Compensation: Can you take time off from work to complete the study requirements or participate solely during non-duty hours? Can you receive compensation for your participation in this study? Will your supervisor give you permission to participate during work hours? See the NIH Policy Manual 2300-630-3 "Leave Policy for NIH Employees Participating in NIH Medical Research Studies."

E. Consent Process: Is the person obtaining your consent for the study your supervisor, a subordinate, or co-worker? If so, is there an independent person monitoring the consent process? If the study PI is a supervisor and intends to obtain consent from you, an independent person (e.g., through Bioethics or the NIMH Human Subjects Protections Unit [HSPU], or others as approved by the IRB) must monitor the consent process. If the person obtaining consent from you is a co-worker then an

independent person (e.g., through Bioethics or the NIMH HSPU, or others as approved by the IRB) may be required to monitor the consent process, as determined by the IRB for the specific study.

If you are thinking of enrolling as a subject at the NIH Clinical Center and you have any questions or concerns, please contact the Office of Human Subjects Research Protections (OHSRP) at (301) 402-3444 and/ or the Patient Representative if you are thinking of enrolling as a subject at the NIH Clinical Center on (301) 496-2626. If you are at a NIH site outside the Clinical Center then please contact local site leadership.

National Heart, Lung, and Blood Institute

Name	
MRN	
DOB	
Genotype	

Protocol 20-H-0080: Red Cell Half Life Determination in Patients with and without Sickle Cell Disease
Schedule of Events: SCD Pre-transplant Patients
Turquoise: To be done after 20-H-0080 protocol signed.

Procedure: Consent Forms	**Screening Eval**	**Day -1**	**W 1, Day 0**	**W 1.5**	**W 2**	**W 2.5**	**W 3**	**W 4**	**W 5**	**W 6**	**W 7**	**W 8**	**W 9**	**W 10**	**W 11**	**W 12**	**W 24 (6 mos)**
20-H-0080 Eligibility checklist																	
20-H-0080 Protocol consent																	
Procedure: History & Physical	**Screening Eval**	**Day -1**	**W 1, Day 0**	**W 1.5**	**W 2**	**W 2.5**	**W 3**	**W 4**	**W 5**	**W 6**	**W 7**	**W 8**	**W 9**	**W 10**	**W 11**	**W 12**	**W 24 (6 mos)**
Medical history																	
Physical examination																	
Vital signs, height/weight																	
Interim illness update																	

Procedures/ Consults:	Screening Eval	Day -1	W 1, Day 0	W 1.5	W 2	W 2.5	W 3	W 4	W 5	W 6	W 7	W 8	W 9	W 10	W 11	W 12	W 24 (6 mos)
DTM for Consult and Venous Assessment																	
DTM for Blood Collection (total vol: ~ 80 mL)																	
VADD Service for 18G or 20G IV (ONLY IF NEEDED)																	
Infusion of Biotinylated RBCs (max vol 50 mL)																	
Procedure: Blood for Clinical Laboratory Testing	**Screening Eval**	**Day -1**	**W 1, Day 0**	**W 1.5**	**W 2**	**W 2.5**	**W 3**	**W 4**	**W 5**	**W 6**	**W 7**	**W 8**	**W 9**	**W 10**	**W 11**	**W 12**	**W 24 (6 mos)**
CBC w/diff																	
Reticulocyte count																	
Hemoglobin electrophoresis																	
Type and antibody screen																	
DAT																	

Acute care panel (CMP)																	
Mineral panel (CMP)																	
Hepatic panel (CMP)																	
LDH																	
B-HCG serum pregnancy (females only)																	
Urinalysis																	
HIV-1/HCV/HBV NAT																	
HBsAg																	
HCV antibody																	
HTLV I/II																	
HIV 1/2																	
HB core antibody																	
West Nile Virus																	
T. cruzi antibody																	
Treponema palladium (antibody) or equivalent (such as RPR)																	

Blood for Research Samples	Screening Eval	Day -1	W 1, Day 0	W 1.5	W 2	W 2.5	W 3	W 4	W 5	W 6	W 7	W 8	W 9	W 10	W 11	W 12	W 24 (6 mos)
α-Globin Genotype Analysis (LVV 3mL tube, 3 mL volume) Alexis pick up for OJ																	
Biotin Antibody Screen (LVV 3mL tube, draw 1 mL vol) Alexis Leonard pick up *will be done during the course of the study if bioRBC degradation is faster than predicted																	
Bluebird Bio Sample (LVV 3mL tube, draw 1-3 mL) Alexis pick up for OJ																	
Eaton Lab (LVV 3mL tube, draw 1 mL) Qaun Li pick up																	
Tisdale Lab (LVV 3 mL tube, draw 1-3 mL) Alexis pick up																	

National Heart, Lung, and Blood Institute

Name	
MRN	
DOB	
Genotype	

Protocol 20-H-0080: Red Cell Half Life Determination in Patients with and without Sickle Cell Disease

Schedule of Events: HbAA, HbAS, and SCD Post-transplant Volunteers

Turquoise: To be done after 20-H-0080 protocol signed.

Procedure: Consent Forms	**Screening Evaluation**	**Day -1**	**Week 1, Day 0**	**Week 2**	**Week 3**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**	**Week 14**	**Week 16**	**Week 24 (6 mos)**
20-H-0080 Eligibility checklist													
20-H-0080 Protocol consent													
Procedure: History & Physical	**Screening Evaluation**	**Day -1**	**Week 1, Day 0**	**Week 2**	**Week 3**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**	**Week 14**	**Week 16**	**Week 24 (6 mos)**
Medical history													
Physical examination													
Vital signs, height/weight													
Interim illness update													
Procedures/ Consults:	**Screening Evaluation**	**Day -1**	**Week 1, Day 0**	**Week 2**	**Week 3**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**	**Week 14**	**Week 16**	**Week 24 (6 mos)**
DTM for Venous Assessment and Consult													
DTM for Blood Collection (total vol: ~ 80 mL)													

VADD Service for 18G or 20G IV (ONLY IF NEEDED)													
Infusion of Biotinylated RBCs (max vol 50 mL)													
Procedure: Blood for Clinical Laboratory Testing	**Screening Evaluation**	**Day -1**	**Week 1, Day 0**	**Week 2**	**Week 3**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**	**Week 14**	**Week 16**	**Week 24 (6 mos)**
CBC w/diff													
Reticulocyte count													
Hemoglobin electrophoresis													
Type and antibody screen													
DAT													
Acute care panel (CMP)													
Mineral panel (CMP)													
Hepatic panel (CMP)													
LDH													
B-HCG serum pregnancy (females only)													
Urinalysis													
HIV-1/HCV/HBV NAT													

HBsAg													
HCV antibody													
HTLV I/II													
HIV 1/2													
HB core antibody													
West Nile Virus													
T. cruzi antibody													
Treponema palladium (antibody) or equivalent (such as RPR)													
Blood for Research Samples	**Screening Evaluation**	**Day -1**	**Week 1, Day 0**	**Week 2**	**Week 3**	**Week 4**	**Week 6**	**Week 8**	**Week 10**	**Week 12**	**Week 14**	**Week 16**	**Week 24 (6 mos)**
α-Globin Genotype Analysis (LVV 3mL tube, 3 mL volume) Alexis pick up and deliver to OJ													
Biotin Antibody Screen (LVV 3mL tube, draw 1 mL vol) Alexis Leonard Pick-up *will be done during the course of the study if bioRBC degradation is faster than predicted													
Bluebird Bio Sample (LVV 3mL tube, draw 1-3mL) Alexis pick up and deliver to OJ													
Eaton Lab (LVV 3mL tube, draw 1 mL) Qaun Li Pick-up													

Tisdale Lab (LVV 3mL tube, draw 1-3mL) Alexis pick-up													