

A NON-INVASIVE METHOD FOR MONITORING TRANSPLANTED ORGAN STATUS IN ORGAN-TRANSPLANT RECIPIENTS

5 **FIELD OF INVENTION:**

The invention pertains to the field of medical diagnostics with special reference to non-invasive method for monitoring transplanted organ status in organ-transplant recipients.

BACKGROUND:

10 Transplantation is a life-extending medical procedure and the most preferred treatment option for end-stage organ damages as long-term mortality is almost 68-70% lower in patients receiving an organ-transplant than those patients who did not receive a transplant. Frequent monitoring of a transplanted graft is difficult as it requires highly invasive procedures like tissue-biopsy, regarded as the “Golden Standard” for determining rejection
15 status of transplant graft and confirmation of graft-rejection, the method is highly invasive necessitating expensive hospitalization, and also carries significant medical risks, including internal bleeding and biopsy-induced transplant graft rejection. An accurate and timely diagnosis of organ transplant rejection in an organ transplant recipient is essential for survival of the organ transplant recipient. Also, the conventional method for detection of
20 rejection is difficult, expensive, and risky, and do not have adequate specificity and sensitivity to elucidate the rejection status of a transplanted organ. In addition, this method suffers from many problems including high costs, variability in tissue biopsy between physicians, and severe patient discomfort.

25 Medical management of transplantation is one of the most challenging and complex specialities in modern medicine. Not all transplanted organ survives in the new body of a transplant recipient and thus, in a few cases, the organ gets rejected by the transplant-recipient, thereby creating critical, life-threatening medical emergency conditions. The important challenge for medical management in transplants is the management of graft-
30 rejection that occurs mostly due to immunological response of the recipient’s body against the transplanted organ. The hyper-acute transplant failure or hyper-acute transplant rejection caused due to rapid immunological response, accounts for a medical emergency and it is imperative to immediately remove the transplanted organ or tissue from the recipient or the acceptor. However, several precautionary measures are incorporated in the standard

operating protocol (SOP) for transplants to minimize the chances of hyper-acute transplant rejection, or transplant failure that includes serotyping and HLA cross-match, in order to determine the most appropriate donor- recipient/acceptor match and the use of combination of immunosuppressant drugs post-transplant.

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In order to overcome several such limitations, with significant advancements in the area of genomics, non-invasive methods have been used, such as a method for measuring gene expression signals which tend to increase when organ transplant rejection occurs, a method for measuring the level of immune proteins, and the like. However, these methods also pose
10 limitations as they tend to produce high false positive results due to the complex cross-reactivity of various immune responses and are based on tissue-specific gene expression signals. With the identification of donor-derived cell free-DNA (ddcfDNA) in the urine and blood of organ transplant recipients, methods have been proposed for non-invasive diagnosis of organ transplant rejection. However, the ddcfDNA is present in minute
15 quantity, whereas the background DNA is present in abundance and poses a limitation to be used as a tool. Thus, a highly specific and sensitive method for analysing this ddcfDNA is required.

The present invention resolves the problems and risks of conventional tissue-biopsy method
20 and the method is highly sensitive and specific for diagnosis of the status of a transplanted organ based on presence of the said one or more circulating nucleic acids markers.

OBJECT OF THE INVENTION:

It is an object of the present invention to provide a non-invasive method for monitoring
25 status of the transplanted organ in organ-transplant recipients by determining the ratio of donor derived markers sequences to the marker sequences of the transplant recipients from circulating cell free DNA of the transplant recipients.

Another object of the invention is to determine a normalized threshold value of the total
30 circulating cell free DNA in healthy and clinically stable and post transplantation patients.

Another object of the invention is the profiling of the nucleic acids of the donor and the recipients and identification of markers.

DRAWINGS:

Fig 1 is a flow chart which depicts the steps of the invention for monitoring the status with the total ccfDNA of the transplant recipients.

Fig 2 is a flow chart which depicts the steps of the invention for monitoring the status with the donor -derived ccfDNA in the transplant recipients.

DETAILED DESCRIPTION:

Accordingly, the present invention provides a non-invasive method for monitoring transplanted organ status in organ-transplant recipients by determining the ratio of donor derived marker sequences to the marker sequences of the transplant recipients from circulating cell free DNA of the transplant recipients using digital droplet PCR.

In one embodiment a normalized threshold value of the total circulating cell free nucleic acids of healthy as well as in post-transplantation patients is determined.

In addition, profiling of the nucleic acids of the donor and the recipients is performed for identification of markers.

The invention provides a method of monitoring the status of a transplanted organ in the transplant recipients, comprising of: (i) collection of sample from a subject who has received an organ transplant from a donor; (ii) identification of the marker sequences from the transplant recipients, wherein one or more nucleic acids that are derived from the donor are identified based on a predetermined profile of markers; and (iii) identification of said one or more marker sequences by analysing the circulating cell free nucleic acids from the transplant recipients by digital droplet polymerase chain reaction (DDPCR); (iv) arriving at a ratio of the donor derived marker sequences to the recipient-derived marker sequences expressed in terms of percent; (v) assessment of the organ status of the transplant recipients on comparison calculated percent with the cut off value.

The cut off value which is the ratio of the donor derived ccfDNA marker sequences to the recipient-derived marker sequences is calculated and expressed as percentage.

The cut off value for monitoring the status of the transplanted organ of the transplant recipient varies with the transplant type; wherein the value is in the range of 0.1% to 2.75%

in heart transplant recipients; <15% in liver transplant recipients and 0.3% to 3.5 % in kidney transplant recipients; wherein the ratio of the donor derived marker sequences to the recipient-derived marker is greater than the cut off value, the recipients organ is considered as in organ rejection state; wherein the ratio of the donor derived marker sequences to the recipient-derived marker is lesser than the cut off value, the recipient organ status is considered as healthy.

The invention provides a method of monitoring the status of a transplanted organ in the transplant recipients, comprising of: (i) collection of sample from a subject who has received an organ transplant from a donor; (ii) quantification of the total circulating cell free DNA in healthy subjects and post transplantation patients; (iii) arriving at a normalised value of the total circulating cell free DNA in healthy subjects and post transplantation patients; (iv) assessment of the organ status of the transplant recipients by comparing the normalised threshold value of total circulating cell free nucleic acids.

A normalized threshold value of the total circulating cell free nucleic acids in a healthy subject is < 7.7 ng/ μ l and <9.63 ng/ μ l in clinically stable and healthy post-transplantation patients.

The normalized threshold value of the total circulating cell free DNA in transplant recipients if greater than 9.63 ng/ μ l, the recipient is considered as in organ rejection state; and wherein the value of the circulating cell free DNA in the transplant recipient is less than or equal to 9.63 ng/ μ l, the recipient organ status is considered as healthy.

The organ-transplant status comprises of graft-rejection, intolerance, allograft injury not amounting to rejection, tolerant healthy transplant function, transplant survival, chronic transplant injury, or pharmacological immunosuppression, all of which is determined by the said non-invasive method. The allograft injury that may not be a rejection, can be ischemic injury, any viral or other pathogenic infections, a reperfusion injury, peri-operative ischemia, chronic hypertension, physiological stress, injuries caused by pharmaceutical agents and injuries due to reactive oxygen species.

The biological sample is selected from whole blood, plasma, serum, saliva or urine.

The marker sequence is a polymorphic genomic marker and is selected from one or more single nucleotide polymorphisms (SNP's), restriction fragment length polymorphisms (RFLP's), short tandem repeats (STRs), variable number tandem repeats (VNTR's), hypervariable regions, minisatellites, microsatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, or insertion or deletion polymorphisms.

The preferred polymorphic marker sequences comprises of one or more of SNPs.

10 The method comprises of genotyping the transplant recipient to determine the donor-derived marker in the transplant recipients. The method further comprises profiling of markers, wherein the donor-derived markers in the transplant recipients is clearly distinguishable.

15 The transplant can be an organ transplant or a skin / tissue transplant.

The organ transplant is selected from one of kidney transplant, heart transplant, liver transplant, pancreas transplant, lungs transplant, intestine transplant, bone marrow or thymus transplant or a combination of more than one organ transplant.

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The marker sequences can be derived from one of circulating cell free DNA, which comprises of double-stranded DNA, or single-stranded DNA, single-stranded DNA hairpins, and DNA/RNA. The nucleic acid can be mRNA also.

25 The amplified marker sequences of total ccfDNA is analysed for the donor-derived marker sequences in the transplant recipients using DDPCR. The method is cost-effective, rapid and absolute quantification of nucleic acids by counting molecules and also have superior analytical precision compared to conventional PCR or qPCRbased detection methods or next generation (NGS) sequencing. The method described herein with the analysis of amplified marker sequences of the donor derived circulating cell free nucleic acid with
30 DDPCR has a specificity between 90% and 100%.

The markers were selected to differentiate the donor-derived ccfDNA from the total ccfDNA in the transplant recipients. The markers of the invention was selected in such way

that it represents sequences spanning the entire human chromosome, including the X and Y chromosomes. The SNP markers selected are those with high minor allele frequency ([MAF > 40] or MAF > 0.4000). For arriving at the candidate 71 SNPs with high Minor Allele Frequency (minor allele frequency [MAF > 40] or MAF > 0.4000) an extensive
5 bioinformatics analysis was performed. Further, the SNPs are selected in such a way that it includes SNPs taken from genes that have been proven to play role in tissue rejection, and cellular necrosis ie, the SNPs with high MAF includes SNPs from those genes that are clinically proven to have a role in tissue rejection and necrosis. Furthermore, the SNPs also includes those SNPs that are proven to help differentiate between two individuals. The final
10 SNPs selected were those having high MAF in both rejection-related genes as well as in the markers that are proven to differentiate between two unrelated individuals, and that spanning across all the chromosomes.

The donor derived ccfDNA amplification is performed by DDPCR for the identification of
15 the organ transplant status in the transplant recipient. Firstly, screening of all the High-MAF SNPs was done in both genomic DNA as well as the circulating cell-free DNA using Real-Time PCR. In this step, the SNPs that have heterozygous genotype in the recipient are filtered out and eliminated, since they cannot be used for quantification in DDPCR. In the next step, the pre-amplified ccfDNA is used as the template, and the candidate SNPs are
20 filtered out and the assay is set for the individual patient. The SNPs which are homozygous in the recipient and that could be either heterozygous or homozygous on the allele in the graft and is preferably heterologous between the recipient and the graft is chosen as the final candidate SNPs. This step is performed as a DDPCR assay.

25 All homozygous SNPs were subsequently used to genotype the recipient's ccfDNA enabling the measurement of the donor derived ccfDNA fraction by using a hydrolysis -based SNP assay in combination with DDPCR. The number of candidate SNPs obtained ranged between 2 and 9 with a minimum of 3 SNPs per patient in the above method. The average of the 3 SNPs determined is measured as absolute copy numbers (copies/ μ l) and can be
30 expressed as percentage of donor-derived ccfDNA markers to the recipient derived markers.

The candidate marker sequences for monitoring the organ status are selected from the set of 71 SNPs with the rf numbers and their location in the chromosome with high Minor Allele

Frequency (minor allele frequency [MAF > 40] or [MAF > 0.4000] is as provided in Table 1. The primers for amplification of circulating cell free nucleic acid are provided in table 1.

5 Table 1- Set of markers of the invention:

Sl. No.	rs IDs	Gene	C hr	MAF	Type	Forward primer	Reverse primer
1	rs2493132	AGT	1	0.4884	SNP	AAAGACGCT GGGATTGGA CA	GTCTCCCTTC GAAAGAGAG C
2	rs6667487	RHOU	1	0.4988	SNP	TCATTGAAG TCAGGCTGT GC	GGTGAGCAAC TTGGAAGCTC
3	rs55392176 4	IL10	1	0.4982	INDEL(Insertio n/deletion), upstream variant	TCCTCACCT ACTGTACAC C	ACACAGGGA GGATGAGTGA T
4	rs4233335	KYAT3	1	0.4946	SNP	ATTCTGGTCT CCGCTGTTTC	GTGAACAGAA CCTCAGGTCT
5	rs10900556	REN	1	0.4425	SNP	GTCTGGAAT CATGGGCAA GT	ACCTAGGGGA TGCTAAGGTT
6	rs1449265	ITGA4	2	0.4892	SNP transcript variant	CTGAGAGGC ACTCATGTG GA	AAGAAGGAG GCAATGCAGA A
7	rs7603052	PDCD1	2	0.4692	SNP	GAAAAGGGT TGAGCCTGT CA	TCATCACGGG TACTGTGAGC
8	rs1317808	MSH2	2	0.4973	SNP	GCCACCTTA GCCTCCCAA AG	AGGGTGACTG TATTAATTAT TGTTCAAAC
9	rs2229813	COL4A4	2	0.493	SNP Missense Variant	TCCAGTCCTT TGTCTCCAG A	AGTGC GGCT GAAAGAAAT A
10	rs4851521	IL1R2	2	0.493	SNP	CACCCACAA CTCTGCTGTA A	GATGTTCTGG ATCCCTCAGC

11	rs7653603	MED12L	3	0.499	SNP transcript variant	AGGGGAAAA AGTCAAAGG CA	CCTTTCCCGA GTATGCACTT
12	rs6791557	TGFBR2	3	0.497	SNP Missense Variant	GAAGATATG GGTTGGGTG CA	GTCTTCCAAC ACCCATGCTA
13	rs7652776	CNTN4	3	0.4645	SNP	GAGGGCTTT GGAGTGGAA AT	TCCAAGGTTT GCTCAAGAGG
14	rs9131	CXCL2	4	0.4387	SNP - 3'UTR Variant	CTGTTGTGCA GTCAGCTTTC	CATACATTTT CCTGCCGTCA
15	rs352007	PF4	4	0.1931	SNP	AGCAAATGC ACACACGTA GG	ATTTGCAAAC CCAAGGACTG
16	rs1818782	DAB2	5	0.4974	SNP	CCAAGCACA GGGTCTCATT T	GAAACCCTTC TTCCCTCTGG
17	rs4701997	DNAH5	5	0.4878	SNP Missense Variant	TGTGGTTAAT CTCTGGGGA T	AGCCCTACAG TTTGACCCTA
18	rs251022	PCDHGA10	5	0.4357	SNP- 500B Downstream Variant (VNTR)	ACACACACA CACGCAATT CGG	ATGAGCTGAG GTGGGTGCTG
19	rs714459	SNX18	5	0.4922	SNP	GGCAGCAAT GTCATTACA GC	GGCTCATTTT AGATCTGGCT
20	rs1801020	SLC34A1	5	0.4724	SNP - 5'UTR Variant	CCACTTGGCT TTCCACAAA C	CGATCTGTTG CTAGTCTGCA
21	rs2842949	TPMT	6	0.4149	SNP	TGCTTGTGTA TGTGAAGGC A	GCCTTTGCCT GTGTAGAGAA
22	rs657941	TAB2	6	0.4988	SNP	CAAACCTCCA GTGTTGGCA TG	CACCCCAAAG CACTCTGTTA
23	rs805294	C6orf25	6	0.4986	SNP	GTTCCCTCTTG CCACACTCTT	AATGTCAGCT GGGAAGACA C

24	rs2523860		6	0.4872	SNP	CAGCCTCTG GTTCCAGGC CT	GGAGAATCCC AGAAGCAGG CTGA
25	rs2766535	FKBP5	6	0.4641	SNP	AACTTAGCT GCTCTTGCTT CAGT	GTACCTGCCT TAACTCAGTA TGATCTT
26	rs1554497	SDK1	7	0.4998	SNP	ATTGCCAAT GTTGGAGGT GT	CTTGGGCTGC TTACAGAAGG
27	rs35024671	UMAD1	7	0.4986	INDEL(Insertion/deletion)	CTTCACTTGG CTTCCTCCTT	TGTGCAGGTT TCAAGGGATT
28	rs7794745	CNTNAP2	7	0.4946	SNP	CAGAAAGGC AGAAATCGG GA	AGCAGCCTTT CAACACTGAT
29	rs15775	KLHL7	7	0.4203	SNP	ATGCATCTCT CTAAGCCCC T	GGCAAAGCAT TGTCGTAACA
30	rs2002792	IL6	7	0.4894	SNP	GGTCAGGAG TTCAAGACC CG	CCTTCCCTGT GCATGGTGAT
31	rs2575694	CTHRC1	8	0.4782	SNP	AATTTGTGTC TCCATCGCC A	CAATCCCCTC TCCCAAGTTG
32	rs756627	RECQL4	8	0.4006	SNP Missense Variant	TGCTTGTGG AGTTCAGTG AG	CACATCAGGC TTCCTCTGAG
33	rs1800392	WRN	8	0.4712	SNP	TGTTAATCTG GTGCCTTGC A	CCACTATGAG CAACGGAGA G
34	rs11794980	LCN2	9	0.4792	SNP	CCATCTCTCC CTCCCAAGG A	CCGAAGTCAG CTCCTTGTT
35	rs10738924	DCAF12	9	0.474	SNP	CAGCACTTT AGGAGGCCA AG	GGTTCAAGCG ATTCTTCTGC
36	rs1197943	LINGO2	9	0.4515	SNP	CTTCCCTTGC CCCTCTTCCA	TGCTCTGTGG ATCCCTGGAG
37	rs4405241	PAX2	10	0.4996	SNP	CTCCCCATG GATATGCAC TG	AATTGTCCTG ACACTGAGGC

38	rs2704522	COL13A 1	10	0.4944	SNP	TGCCATCAT ATGCATGCA GA	ATGAAATTCA CCTGCCCACA
39	rs1512705	CUBN	10	0.4972	SNP	TGGATCTGC CACTCAAAG TG	TTTTAGCTCT GCCATCTCGG
40	rs10734083		10	0.4916	SNP	GGCATCTGA ATTCAAGCTT TGGTC	TTCTTCTAGTT GGTCTGGTAG GCT
41	rs1073525	M- ARCH8	10	0.4533	SNP	ACTCAGAAA GGTGGGAGG AT	CCCTCACTTC GGTCAGTTTT
42	rs668393	KCNJ1	11	0.4986	SNP	AGGTCTGCC ATGTGAATG AC	GCCATCAGCT AAGGTCTCTG
43	rs1522662		11	0.4816	SNP	ACCGTGACC CTCAGTTCCT T	AAGAGCCCTT ATAAGGTGTG AGAAA
44	rs481235	SLC3A2	11	0.4872	SNP	CGCCTGTAA TCCCAACAC TT	AGAGTGCAAT GGCTCGATCT
45	rs2277312	SLC22A 11	11	0.4531	SNP	GAGGCCTAT GACCATCTG GC	GCAGAGCCAG GTCACATTCT
46	rs2043055	IL18	11	0.4806	SNP	CCACCTGAA AGCCAATGA GA	CCACCTGAAA GCCAATGAGA
47	rs10841697	SLC01B 3	12	0.4946	SNP	GCTTTTCCAG GCACACAGT G	TTGGGCTTAT GAGTGGGCAG
48	rs657197	ATXN2	12	0.4687	SNP	CACTCACCCCT TGTGGACCTT	GCTGATGCAA AATCAAAGCA
49	rs9554250	FLT3	13	0.498	SNP	TGTGAGCCA TCCAAAACC TT	AGCAATGTGT ACTGTGGCTT
50	rs7328030	LINC023 37	13	0.4673	SNP	CAGCCAATT TCTTCCCTGG A	AGGACTGGAA AACGTGACAG

51	rs1626923	SPTB	14	0.4451	SNP	TTTTGCAGTG GGTAGGACA G	ACTGGCTTGA GCTTTCCATT
52	rs978511		14	0.48	SNP	GGCATGTTG ATGGATGGG AT	TTGCTGACAG TAGAACTCGC
53	rs741761	SEMA7 A	15	0.495	SNP Missense Variant	TGCTGATCCT CTTTCGTCCT	TCCCTCAGAC CAGGACAATC
54	rs10162971	NR2F2- AS1	15	0.4964	SNP	ACTATCAAG CCCACAGGA GA	GCTTAGATGA GGCCTTCTGG
55	rs12449089	ITGAM	16	0.4573	SNP	GCCCAGCTT GAATTGACC TA	CAGCTAAATG GGAGGCTGAG
56	rs7193058	UMOD	16	0.4781	SNP	CACTCACAG TGCCATCCAT C	CCATTCCTGG AGCTCACAAC
57	rs11866418	SOX8	16	0.4808	SNP 3'UTR Variant	TCTACCAGT ACCCCTGCTT C	GAGGGAATGT GGCCTTGAG
58	rs4323	ACE	17	0.4872	SNP	AGGCCAACT GGA ACTACA AC	CTTCAAGTGA TCCTCCCACC
59	rs2159132	COX10	17	0.4918	SNP	CACAATGAC AAGCTCAGG GA	TTCTGGCCTT TACAGTTGGG
60	rs150384	NEDD4 L	18	0.4998	SNP	GCGATGATG TCTCTGAGG CA	ACAGACCCTG CCCACAAAAA
61	rs3909244	DLGAP1	18	0.4898	SNP	TGGTTAAAC TG TAGTACA TCCATGGA	ACCTTTTGGG ACTGGCTTTC T
62	rs12459052	SLC7A9	19	0.4956	SNP	TCCACATGTC AGGTGTCTG C	GAGATGGGGT TTCACCGTGT
63	rs67233828	TGFB1	19	0.4878	INDEL(Insertio n/deletion), Intron variant	CTAGGCTCCT ACAATGTGC C	GCCGAGGTGG GTGGAT

64	rs3918251	MMP9	20	0.4938	SNP	GAGTCGAAA TCTCTGGGG CC	GGACACCCCA TATCGCAGAG
65	rs2327088	PLCB1	20	0.4517	SNP	CCGATGCTG CTGAGGATA AA	TCCAGTGTGC AAAGACTCTG
66	rs1051266	SLC19A 1	21	0.4886	SNP Missense Variant	TGGCCCCAA ACCCTAAAT TT	CCGTAGAAGC AAAGGTAGCA
67	rs2742630	UPK3A	22	0.4986	SNP - 2KB upstream variant	CCTTGCCCCT CTTCTGTTGT	CCCACTTGGA GCCTCAGTTT
68	rs1801198	TCN2	22	0.4203	SNP Missense Variant	TGTTGCCCTT CTTCTCCAAG	AGGTCTTGTG GTTCAGAACG
69	rs6609533	TIMP1	X	0.4734	SNP Missense Variant	AATGGTCCC ACTGGAAAT GG	TTAGGGAACA GCACTTTGGG
70	rs2298065	EFHC2	X	0.4185	non-coding Transcript Variant	CATCCAAAG GTGGCACTT GC	ACGTTGCAGC CATACAGACA
71		SRY	Y			TTGTGCAGC CATCACCTCT	AAATCAGATT AATGGTTGCT

The transplant organ status of the transplant recipients was monitored until the rejection outcome and the frequency of monitoring the status is determined based on the transplant organ and the ratio of cut off values over time.

EXAMPLES:

The invention is illustrated by various examples which are not meant to limit the scope of the invention in any manner. All the embodiments that may be obvious to a skilled person in view of the disclosure would fall within the scope of the present invention.

Example 1:

STEP-1: Sample collection and Separation of plasma

5 ml of peripheral blood was collected by venipuncture into an EDTA-coated collection tube both from the donor and the recipient.

Urine samples were prepared by allowing it to stand until all the debris is settled and the clear upper layer is taken for analysis.

From this, 2 ml of blood was centrifuged at 5000 rpm for 10 min and plasma was carefully removed from the top and stored at -20°C for further use.

STEP-2: Isolation of circulating cell-free DNA, Genomic DNA:

2(a) Isolation of circulating cell-free DNA (ccfDNA):

The circulating cell-free DNA (ccfDNA) is isolated from plasma following the manufacturer's instructions of EpiQuik Circulating cell-free DNA Isolation kit, Epigentek Group Inc. USA. The ccfDNA was isolated by adding 0.5 ml of plasma with 24µl of ccfDNA capture Enhancer; 900µl of Capture Buffer and 50µl ccfDNA Capture Beads into 1.7ml micro centrifuge tube. The solution was mixed well repeated pipetting atleast 20 times and incubated at room temperature for 10 minutes. The solution was centrifuged at 12000 rpm for 5 minutes. The DNA release solution was prepared by mixing 2 µl of proteinase K with 40 µl of digestion solution. The supernatant was discarded and 40 µl of DNA release solution is added to the tube and beads are resuspended, incubated at 55°C for 10 minutes. The tubes were placed on a magnetic stirrer and the supernatant containing the DNA was transferred to a tube containing MQ binding beads and 2X bead solution. It was mixed thoroughly by pipetting atleast 10 times and incubated for 5 minutes at room temperature to allow the DNA to bind to the beads. Again, the tube is placed on a magnetic stand and the beads are resuspended in 200 µl of ethanol. The step was repeated twice, and ethanol was removed by airdrying. The DNA was eluted with 20 µl of elution buffer, and finally the cell free DNA was isolated.

2(b) Isolation of whole Genomic DNA:

The Genomic DNA was isolated using Mag Attract Blood DNA Kit, Qiagen, USA, and the protocol followed was as per the manufacturer's instructions as given below: 500µl of blood sample is taken in a sterile micro centrifuge tube and 1 ml of Blood Lysis Buffer – 1 was added. 50µl of solution A was added and the contents were mixed by inverting the tube for 4-5 times and incubated at room temperature for 5 minutes. The samples were centrifuged at 8000 rpm for 5 minutes. Supernatant was discarded and the pellet was resuspend in the Blood Lysis Buffer – 1 by pipette mixing. The samples were centrifuged at 8000 rpm for 5 minutes and the previous steps were repeated. 20µl of RNase A Solution was added to the resuspended pellet and incubated at room temperature for 15 minutes; followed by addition of 20µl of Proteinase K. The sample was incubated at 56°C for 10 minutes. To the lysate 350µl of Blood Magna Mix - 3 was added and the contents are mixed by inverting the tube

for 10 – 12 times. The lysate was incubated at room temperature for 5 minutes and placed on magna stand for 2 minutes or until solution appear clear. The supernatant was discarded without distributing the pellet. 500µl of Blood Wash Buffer – 4 was added to the pellet and repeatedly washed with resuspended in blood wash buffer 5 to get the pellet which is then
5 airdried. The pellet was resuspended in nuclease free water, incubated at 65°C for 5 minutes. The tube is allowed to stand and the supernatant containing the DNA was isolated.

2(c) Quality Check through Quantification of Isolated DNA:

The isolated DNA (Cell-Free DNA or Whole Genomic DNA) are quantified for the
10 concentration and quality by Nano-Drop as per the protocol given below:

1µl of isolated DNA (Cell-free DNA or Whole Genomic DNA) is placed on the Nano-Drop Spectrophotometer (Thermofisher Scientific) and the ratio of the readings at 260nm and 280 nm is used for quantifying the isolated DNA.

Example 2:

PCR amplification and determination of total ccfDNA value:

Total ccfDNA was quantified using multi locus LIPA2 regions. LIPA2 is a human Long Interspersed Element (LINE) of the class L1, that is well interspersed throughout the human genome. Reaction mixture for each LIPA-qPCR (90 bp and 222 bp amplicons) contained
20 3µl DNA template, 0.5 µl of the each forward and reverse primer, 0.4 µl Rox as passive reference dye, 10 µl SYBR Green Master Mix (KAPA) and made up to total reaction volume of 20 µl with 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, and annealing at 64 °C for 1 min in Stepone plus Real-Time PCR System (Applied Biosystems, USA).

25 LIPA-qPCR reactions were standardized using 10 healthy control samples to yield the total ccfDNA results. The “control-value” is an average of total ccfDNA value of the ccfDNA levels of 10 healthy control and used as the standard. “Control Value” of the total ccfDNA of healthy subject is < 7.7 ng/µl. The total ccfDNA of clinically stable and healthy post-transplant subjects was found to be less than 9.63 ng/µl and the total ccfDNA value above
30 9.63 ng/µl in a is considered to be significantly high.

In addition to this, melting curve was performed after each PCR reaction to ensure the amplification of a single peak for all samples. The integrity index is determined as the ratio

of long fragments to that of short fragment of DNA. The Integrity Index of the DNA confirms the girth or the density of the DNA.

Example 3:

5 PCR amplification and determination of the donor-derived ccfDNA.

The donor derived ccfDNA amplification is performed by DDPCR for the identification of the organ transplant status in the transplant recipient. Firstly, screening of all the High-MAF SNPs was done in both genomic DNA as well as the circulating cell-free DNA using Real-Time PCR. In this step, the SNPs that have heterozygous genotype in the recipient are
10 filtered out and eliminated, since they cannot be used for quantification in DDPCR. In the next step, the pre-amplified ccfDNA is used as the template, and the candidate SNPs are filtered out and the assay is set for the individual patient. The SNPs which are homozygous in the recipient and that could be either heterozygous or homozygous on the allele in the graft and is preferably heterologous between the recipient and the graft is chosen as the final
15 candidate SNPs. This step is performed as a DDPCR assay.

All homozygous SNPs were subsequently used to genotype the recipient's ccfDNA enabling the measurement of the donor derived ccfDNA fraction by using a hydrolysis based SNP assay in combination with DDPCR. The number of candidate SNPs obtained ranged
20 between 2 and 9 with a minimum of 3 SNPs per patient in the above method. The average of the 3 SNPs determined is measured as absolute copy numbers (copies/ μ l) and is expressed as percentage of donor-derived ccfDNA markers to the recipient derived markers. The percentages and numbers of assays given for each selection step are calculated for a minor allele frequency of 0.5 and can vary between individual patients. The percentage of donor-
25 derived ccfDNA differs significantly between various organ transplants as given below.

Type of transplant (Tx)	Mean Cut-off Value of Donor-Derived ccfDNA in the transplant recipients	Variability (in terms of values)	Outcome
Heart Tx	0.1% to 3.0%	+/- 0.9%	Healthy Tx Patients

Heart Tx	2.75% to 4.55%	+/- 1.8%	Acute Rejection in Heart Tx
Liver Tx	Less than 15%	+/- 5%	Healthy Liver transplant
Liver Tx	30% - 60%	+/-10%	Liver Transplant Rejection
Kidney Tx	0.3 to 3.5%	+/- 1.1%	Healthy Kidney Tx
Kidney Tx	Over 3% to 5.5%	+/-1.5%	Acute Rejection

Example 4:**SNP Selection:**

- 5 For arriving at the candidate 71 SNPs with high Minor Allele Frequency (minor allele frequency [MAF > 40] or MAF > 0.4000 an extensive bioinformatics analysis was performed. It includes the selection of the genes that are proven to play role in tissue rejection and necrosis as well as the SNP markers that are proven to help differentiate between two individuals. The SNPS having high MAF in both rejection-related genes as
- 10 well as in the markers that are proven to differentiate between two unrelated individuals, and that is spanning across all the chromosomes is selected.

Example 5:**Sample Validation**

- 15 A total of 19 patients (13 heart transplant, 5 kidney transplant and 1 kidney+ pancreas transplant) was used for validation. From the group of 13 patients who had undergone heart transplant, 11 patients had successful 1 year survival and 2 patients died within 10 days of heart transplant. In 5 kidney and 1 patient who had undergone combined kidney and pancreas transplant had a healthy 1year survival rate. The below is the table of only the total
- 20 ccfDNA in determining transplant outcome and the infection status.

S no	Type of Transplant	No of patients	Actual value	Cutoff value	Outcome prediction
1.	Heart Tx	11	2 – 9 ng/ul	9.63ng/ul	Clinically stable and healthy
2.	Heart Tx	1	15.8 ng/ul	9.63ng/ul	Immediate Rejection, patient expired.
3.	Heart Tx	1	8.5 ng/ul	9.63ng/ul	No rejection found, but patient expired of cardiac arrest.
5.	Kidney Tx	3	6-8 ng/ul	9.63ng/ul	Clinically stable and Healthy
6.	Kidney Tx	1	12.24 ng/ul	9.63ng/ul	High total cfDNA level, Suspected Rejection, elevated creatinine levels, anti-rejection prophylaxis.
7.	Kidney + Pancreas Tx	1	6.8 ng/ul	9.63ng/ul	No Rejection Found. Clinically stable, healthy.

Advantages of the Invention:

The method is cost-effective, rapid and absolute quantification of nucleic acids by counting molecules and also have superior analytical precision compared to conventional PCR or qPCR based detection methods or next generation (NGS) sequencing. The method described herein with the analysis of amplified marker sequences of the donor derived circulating cell free nucleic acid with DDPCR has a specificity between 90% and 100%.

The SNP selected have high MAF, and it includes SNPs taken from genes that have been proven to play role in rejection, ie, the SNPs with high MAF includes SNPs from the genes that are clinically proven to be found playing role in rejection. The selected 71 SNPs covers the entire genome, including X and Y chromosomes. This way of selection minimizes the error rate.

There is no requirement of Donor DNA to detect the donor-derived ccf-DNA and it is done with the transplant recipients.

For both gDNA and ccf-DNA are together screened for eliminating the heterozygous genotype in the transplant recipients. This saves a lot in reagents and time, there by making the assay cost-effective.

The value of total ccfDNA itself is used as marker to evaluate the transplant status and is clinically validated, as elevated total ccfDNA can be a hallmark of rejection or an infection.

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The Claims:

1. A method of monitoring the status of a transplanted organ in the transplant recipients, comprising of: (i) collection of biological sample from a subject who has received an organ transplant from a donor; (ii) identification of the marker sequences from the transplant donor, wherein one or more nucleic acids that are derived from the donor are identified based on a predetermined profile of markers; and (iii) identification of said one or more marker sequences by analysing the circulating cell free nucleic acids from the transplant recipients by digital droplet polymerase chain reaction (DDPCR); (iv) arriving at a ratio of the donor derived marker sequences to the recipient-derived marker sequences expressed in terms of percent; (v) assessment of the organ status of the transplant recipients on comparison of the calculated percent with the cut off value.
2. The method according to claim 1, wherein the markers are selected from one or more SNP markers from the candidate SNP as in Table 1.
3. The method according to claim 1, wherein the markers are selected with high MAF value of $MAF > 40$ or $MAF > 0.4000$.
4. The method according to claim 1, wherein the markers are selected from the entire human chromosome including the X and Y.
5. The method according to claim 1, wherein candidate SNPs obtained ranged between 2 and 9 with a minimum of 3 SNPs per patient and wherein average of the 3 SNPs determined is measured as absolute copy numbers (copies/ μ l) and expressed as percentage of donor-derived ccfDNA markers to the recipient derived markers.
6. The method according to claim 1, wherein the cut off value for monitoring the status of the transplanted organ of the transplant recipient varies with the transplant type and is the range of 0.1 % to 15%.

7. The method according to claim 1, wherein the marker sequence is a polymorphic genomic marker and is selected from one or more single nucleotide polymorphisms (SNP's), restriction fragment length polymorphisms (RFLP's), short tandem repeats (STRs), variable number tandem repeats (VNTR's), hypervariable regions, minisatellites, microsatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, or insertion or deletion polymorphisms.
8. A method of monitoring the status of a transplanted organ in the transplant recipients, comprising of: (i) collection of biological sample from a subject who has received an organ transplant from a donor; (ii) quantification of the total circulating cell free DNA in healthy subjects and post transplantation patients; (iii) arriving at a normalised value of the total circulating cell free DNA in healthy subjects and post transplantation patients; (iv) assessment of the organ status of the transplant recipients by comparing the normalised threshold value of total circulating cell free nucleic acids.
9. The method according to claim 8, wherein the normalized threshold value of the total circulating cell free nucleic acids in a healthy subject is $< 7.7 \text{ ng}/\mu\text{l}$ and $< 9.63 \text{ ng}/\mu\text{l}$ in clinically stable and healthy post-transplantation patients.
10. The method according to claim 9, wherein the normalized threshold value of the total circulating cell free DNA in transplant recipients if greater than $9.63 \text{ ng}/\mu\text{l}$, the recipient is considered as in organ rejection state; and wherein the value of the circulating cell free DNA in the transplant recipient is less than or equal to $9.63 \text{ ng}/\mu\text{l}$, the recipient organ status is considered as healthy.
11. The method according to claims 1 and 8, wherein the biological sample is selected from whole blood, plasma, serum or urine.
12. The method according to claims 1 and 8, wherein the transplant is selected from an organ transplant or a skin / tissue transplant.

13. The method according to claims 1 and 8, wherein the organ transplant is selected from one of kidney transplant, heart transplant, liver transplant, pancreas transplant, lungs transplant, intestine transplant, bone marrow or thymus transplant or a combination of more than one organ transplant.
- 5
14. The method according to claims 1 and 8, wherein the monitoring of the transplant organ status of the transplant recipients comprises of until the rejection outcome.
- 10
15. The method according to claims 1 and 8, wherein the frequency of monitoring of the transplant organ status of the transplant recipients is function of time determined based on the transplant organ and the ratio of cut off values over time.