

Droplet digital polymerase chain reaction for DNMT3A and IDH1/2 mutations to improve early detection of acute myeloid leukemia relapse after allogeneic hematopoietic stem cell transplantation

Chiara Brambati,¹ Silvia Galbiati,² Elisabetta Xue,^{1,3} Cristina Toffalori,¹ Lara Crucitti,³ Raffaella Greco,³ Elisa Sala,³ Alessandra Crippa,⁴ Lorenza Chiesa,¹ Nadia Soriani,² Benedetta Mazzi,¹ Cristina Tresoldi,⁴ Maria Teresa Lupo Stanghellini,³ Jacopo Peccatori,³ Matteo G. Carrabba,³ Massimo Bernardi,³ Maurizio Ferrari,² Vito Lampasona,² Fabio Ciceri,³ and Luca Vago^{1,3}

¹Unit of Immunogenetics, Leukemia Genomics and Immunobiology; ²Genomics for the Diagnosis of Human Pathologies Unit; ³Unit of Hematology and Bone Marrow Transplantation; and ⁴Molecular Hematology Laboratory, IRCCS San Raffaele Scientific Institute, Milano, Italy.

Correspondence: ciceri.fabio@hsr.it
doi:10.3324/haematol.2015.135467

SUPPLEMENTARY METHODS

Patient and Transplant Characteristics

To identify patients carrying mutations in DNMT3A, IDH1, and IDH2 we retrospectively analyzed samples from 89 patients who received allogeneic HSCT at the San Raffaele Scientific Institute between January 2009 and November 2014. As detailed in Table 1, all patients suffered from myeloid malignancies (58 de novo AMLs, 29 secondary AMLs and 2 myelodysplastic syndromes) and received conditioning with myeloablative purpose, based on full-dose Treosulfan ($14 \text{ g/m}^2 \times 3$ days; n=75), intravenous Busulfan (n=12), or Total Body Irradiation (n=2). The majority of patients displayed intermediate-risk cytogenetics (58/89, 65%), were transplanted in the presence of hematologically active disease (53/89, 59%), and from partially HLA-incompatible related donors (50/89, 56%). Unmanipulated G-CSF mobilized peripheral blood was the most common graft source (76/89, 85%), and sirolimus plus mycophenolate the preferred pharmacological GvHD prophylaxis (56/89, 63%). Similar features were documented in the subgroup of patients that resulted positive for the mutations of interest (n=30), and in the patient who were monitored longitudinally during their follow-up (n=17).

Sample Collection and Processing

Bone marrow aspirate samples were harvested in concomitance to routine diagnostic procedures and upon written informed consent approved by the San Raffaele Ethic Committee. Genomic DNA was extracted using the Qiamp Blood Minikit (QIAGEN, Venlo, The Netherlands), checked for purity using a Nanodrop spectrophotometer (Thermo Scientific, Franklin, MA, USA) and stored at -20°C for further analyses. RNA was extracted using the Trizol® reagent (Invitrogen, Carlsbad, CA, USA) followed by a phenol/chloroform precipitation procedure according to the manufacturer's recommendations and stored at -80°C for further analyses: when necessary, it was retro-transcribed

into complementary DNA according to standard published protocols¹ and employed as template.

DNA Sequencing for Mutation Screening

To screen AML samples for the eventual presence of the most common mutations in DNMT3A, IDH1 and IDH2, we designed specific primers employing the OligoAnalyzer (Integrated DNA Technologies, Inc, Coralville, IA, USA) and OligoExplorer (Gene Link™) software.

The table below summarizes primer sequences and genomic coordinates.

Gene	Region	Primer sequence	
DNMT3A	Exon 23 (2937-3114)	For	GTATTTGGTTTCCCAGTCCAC
		Rev	AACTTTGTGTCGCTACCTCAG
IDH1	Exon 4 (2821-3182)	For	GGATGCTGCAGAAGCTATAA
		Rev	TTCATACCTTGCTTAATGGGTGT
IDH2	Exon 4 (1707-1930)	For	ACCACTATTATCTCTGTCCTCAC
		Rev	TGCCCAGGTCAGTGGATC

PCR was performed using 0,062U/μl AmpliTaq Gold® (Applied Biosystems®, Foster City, CA, USA) with 600 nM Forward Primer and 600 nM Reverse Primer. PCR conditions were the same for all the genes: 95°C for 10', followed by 30 cycles of amplification (95°C for 20'', 58°C for 30'' and 72°C for 1'), ending with 72°C for 10'. PCR amplicons were purified using "QIAquick PCR Purification Kit" (QIAGEN, Venlo, The Netherlands) and used for conventional Sanger sequencing.

Droplet Digital PCR Experiments

ddPCR experiments were performed and analyzed in accordance with Bio-Rad Protocols (Bio-Rad Laboratories, Hercules, CA, USA) and with MIQE guidelines for digital PCR². We employed the QX100 Droplet Digital PCR system, testing in duplicate 25 ng of genomic DNA in two reaction wells. Samples were digested for 20 minutes at 37°C using the FastDigest HindIII enzyme (Thermo Scientific, Franklin, MA, USA). Commercial PrimePCR™ ddPCR™ Mutation Assays were available for all tested mutations and respective wild-type counterparts, except for the DNMT3A R882C mutation, for which a custom assay was specifically designed.

Cycling conditions were 95°C for 5', followed by 40 cycles of amplification (94°C for 30'', 55°C for 1'), ending with 98°C for 10', according to the manufacturer's protocol. A minimum of 1'300 and an average of 4'200 template-containing droplets were acquired per each sample, over a total number of approximately 20'000 acquired droplets per samples. Results were analyzed using the QuantaSoft™ software (Bio-Rad Laboratories, Hercules, CA, USA), pooling results from the duplicates, and deriving fractional abundance of each mutation from a Poisson distribution.

Quantitative PCR-based Assays

All qPCR-based assays were performed in accordance with published protocols and with the MIQE and REMARK guidelines^{3,4}.

Briefly, relative abundance of NPM1 mutation A was determined using the *ipsogen*® NPM1 mutA MutaQuant® Kit (QIAGEN, Venlo, The Netherlands) and according to the indications from Gorello and collaborators⁵, employing cDNA as template and ABL1 as reference gene.

Each reaction was performed in duplicates, using 100 ng of RNA equivalent cDNA per well, and always testing in parallel negative templates as control. As cDNA quality control, a minimum of 10'000 copies of ABL were necessary per each reaction well. Results were obtained upon interpolation of data with those obtained from a standard curve. Maximal reproducible sensitivity of this assay is 10^{-4} NPM1^{mut} copies /ABL copy, and maximal sensitivity 10^{-6} NPM1^{mut} copies/ABL copy. In case of positive results approaching the sensitivity limit of the method (i.e. in the range between 10^{-4} and 10^{-6} NPM1^{mut} copies/ABL copy), samples were re-tested in triplicates to confirm the result. Values below 10^{-6} NPM1^{mut} copies/ABL copy were considered negative.

In an analogous manner, WT1 transcript was quantified using the *ipsogen*® WT1 ProfileQuant® Kit (ELN*) (QIAGEN, Venlo, The Netherlands), using cDNA as template and ABL1 as reference gene, following the manufacturer's protocol and the indications published by Cilloni and collaborators⁶. Also in this case, results were obtained upon interpolation of data with those

obtained from a standard curve, starting from the lower reference point of 10 WT1 copies per 10^4 ABL copies. However, given the high background level of this transcript even in the bone marrow of healthy subjects, according to the indications of Cilloni and coworkers 250 WT1 copies per 10^4 ABL copies were considered as the cut-off for WT1 overexpression in the bone marrow.

Host-specific hematopoietic chimerism was assessed using the AlleleSEQR® Chimerism Assay, (Celera Genomics, Alameda, CA, USA), according to the manufacturer recommendations, and using genomic DNA as template. This qPCR-based assay is sensitive enough to detect up to 0.2% host DNA in a chimeric samples. Taking advantage of the $\Delta\Delta C_t$ method, the relative amount of a host-specific marker was assessed by relating the C_t value derived from amplification of that marker to the C_t value of its respective endogenous control (RNaseP) both in pre-HSCT sample (calibrator ΔC_tC) and in post-HSCT chimeric sample (unknown ΔC_tU). In particular, based on our center experience and to published data⁷, we considered as threshold for relapse prediction a host-specific signal above 1%.

SUPPLEMENTARY RESULTS

Validation of Droplet Digital PCR Assays

Droplet Digital PCR assays specific for the DNMT3A R882H, IDH1 R132H and R132C, IDH2 R140Q and R172K mutations are commercially available, and technical validation for sensitivity and specificity has been performed from the manufacturer using appropriate mutant cell line and made available through their website (<http://www.bio-rad.com/>). All these assays were shown to be sensitive and specific up to the 0.1% mutant allele frequency.

The assay specific for the DNMT3A R882C was custom-designed for our study: to validate its technical performance, we tested by ddPCR serial dilutions of genomic DNA from purified DNMT3A R882C-mutant primary AML blasts into wild-type genomic DNA. In five independent

experiments, we demonstrated that the assay allow reliable detection and quantification above the 0.1% mutant allele frequency (maximal reproducible sensitivity), which also represented the maximal background signal for wild-type genomic DNA (Supplementary Figure 1). Analogous results were obtained by cloning the relevant mutation in the pCR™2.1-TOPO® vector (Thermo Scientific, Franklin, MA, USA), and performing serial dilution of this mutation-specific plasmid in wild-type genomic DNA (data not shown).

All six ddPCR assays were further tested for specificity using a series of human genomic DNA samples expected to be negative for the mutations of interest, including peripheral blood from healthy individuals (n=21), bone marrow from healthy individuals (n=10), and bone marrow from AML patients resulted negative for the mutations by conventional Sanger sequencing (n=11). As expected for all these negative controls, all ddPCR assays gave results below the 0.1% mutant allele frequency positivity threshold (Supplementary Figure 2).

SUPPLEMENTARY REFERENCES

1. Gabert J, Beillard E, van der Velden VH, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. *Leukemia*. 2003;17(12):2318-2357.
2. Huggett JF, Foy CA, Benes V, et al. The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. *Clin Chem*. 2013;59(6):892-902.
3. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009;55(4):611-622.
4. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumor marker prognostic studies (REMARK). *J Natl Cancer Inst*. 2005;97(16):1180-1184.
5. Gorello P, Cazzaniga G, Alberti F, et al. Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. *Leukemia*. 2006;20(6):1103-1108.
6. Cilloni D, Renneville A, Hermitte F, et al. Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized WT1 assay to enhance risk stratification in acute myeloid leukemia: a European LeukemiaNet study. *J Clin Oncol*. 2009;27(31):5195-5201.
7. Qin XY, Li GX, Qin YZ, et al. Quantitative chimerism: an independent acute leukemia prognosis indicator following allogeneic hematopoietic SCT. *Bone Marrow Transplant*. 2014;49(10):1269-1277.

SUPPLEMENTARY FIGURE LEGENDS

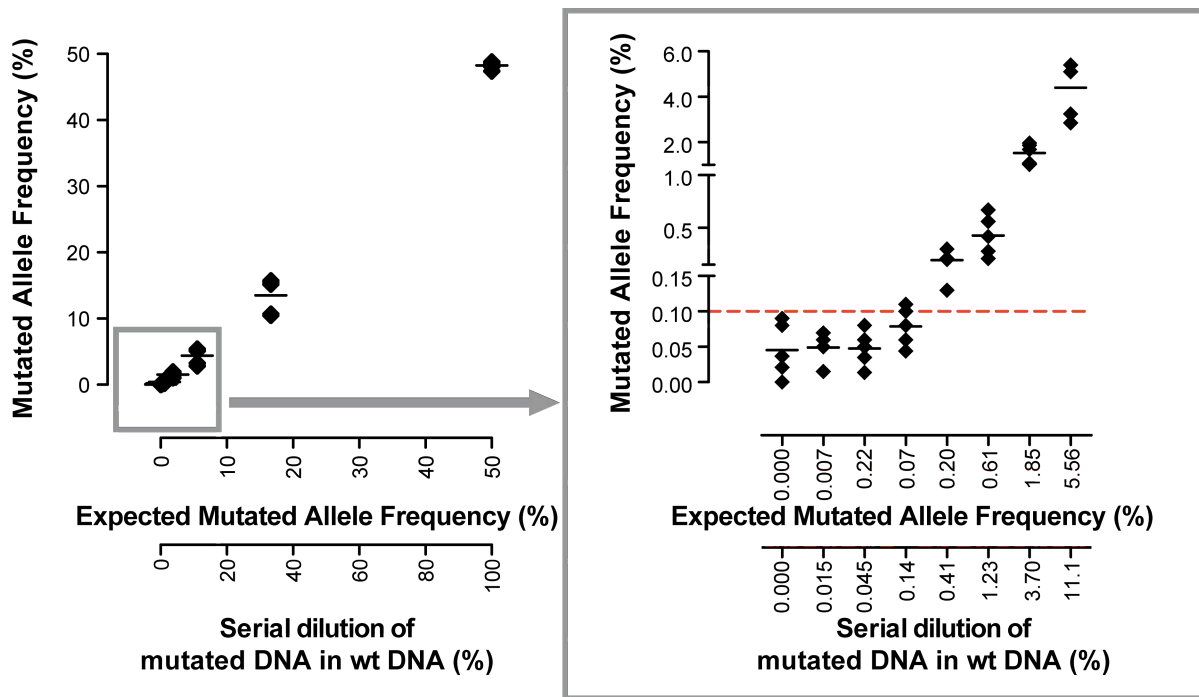
Supplementary Figure 1. DNA dilution curve for technical validation of the DNMT3A R882C mutation-specific ddPCR assay. Genomic DNA extracted from leukemic blasts purified from UPN#68 was serially diluted in genomic DNA extracted from the bone marrow of a healthy individual, and tested with the custom-design mutation-specific ddPCR assay. Shown are results from five independent experiments, each performed in technical duplicates. The dashed red line indicates the 0.1% mutant allele frequency threshold, validated as maximal reproducible sensitivity of the assay.

Supplementary Figure 2. Validation of the specificity of ddPCR assays in expectedly mutation-negative samples. ddPCR results obtained employing the six assays indicated on the x axis to test genomic DNA samples extracted from peripheral blood mononuclear cells of healthy individuals (black squares, n=21), from bone marrow aspirates of healthy individuals (white diamonds, n=10), and from bone marrow aspirates collected at AML diagnosis and resulted negative for the mutations of interest by conventional Sanger sequencing (gray triangles, n=11). The dashed red line indicates the 0.1% mutant allele frequency positivity threshold.

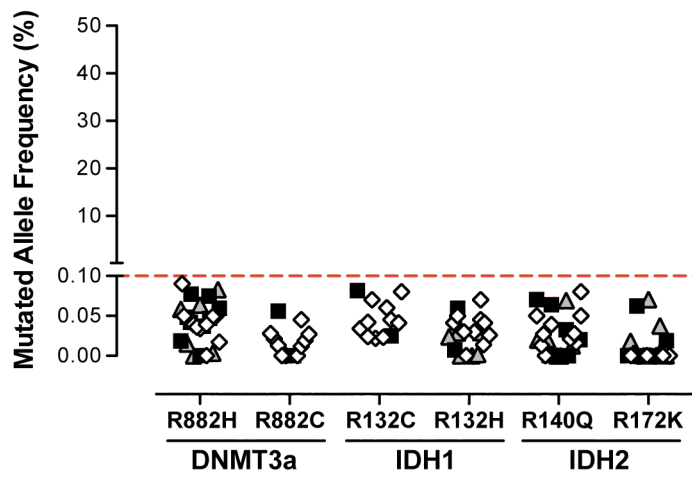
Supplementary Figure 3. Post-transplantation MRD monitoring using different molecular assays in additional relapsed patients. Shown are results obtained during the longitudinal follow-up of 7 relapsed patients employing ddPCR assays specific for mutations in DNMT3A and IDH1/2 (in black) or qPCR assays specific for the WT1 gene transcript (in green), for host-specific chimerism markers (in cyan), or for NPM1 mutation A (in purple). The dashed red line indicates the positivity threshold for all four assays. Time of relapse is boxed in red.

Supplementary Figure 4. Post-transplantation MRD monitoring using different molecular assays in additional non-relapsed patients. Shown are results obtained during the longitudinal follow-up of 6 non-relapsed patients employing ddPCR assays specific for mutations in DNMT3A and IDH1/2 (in black) or qPCR assays specific for the WT1 gene transcript (in green), for host-specific chimerism markers (in cyan), or for NPM1 mutation A (in purple). The dashed red line indicates the positivity threshold for all four assays.

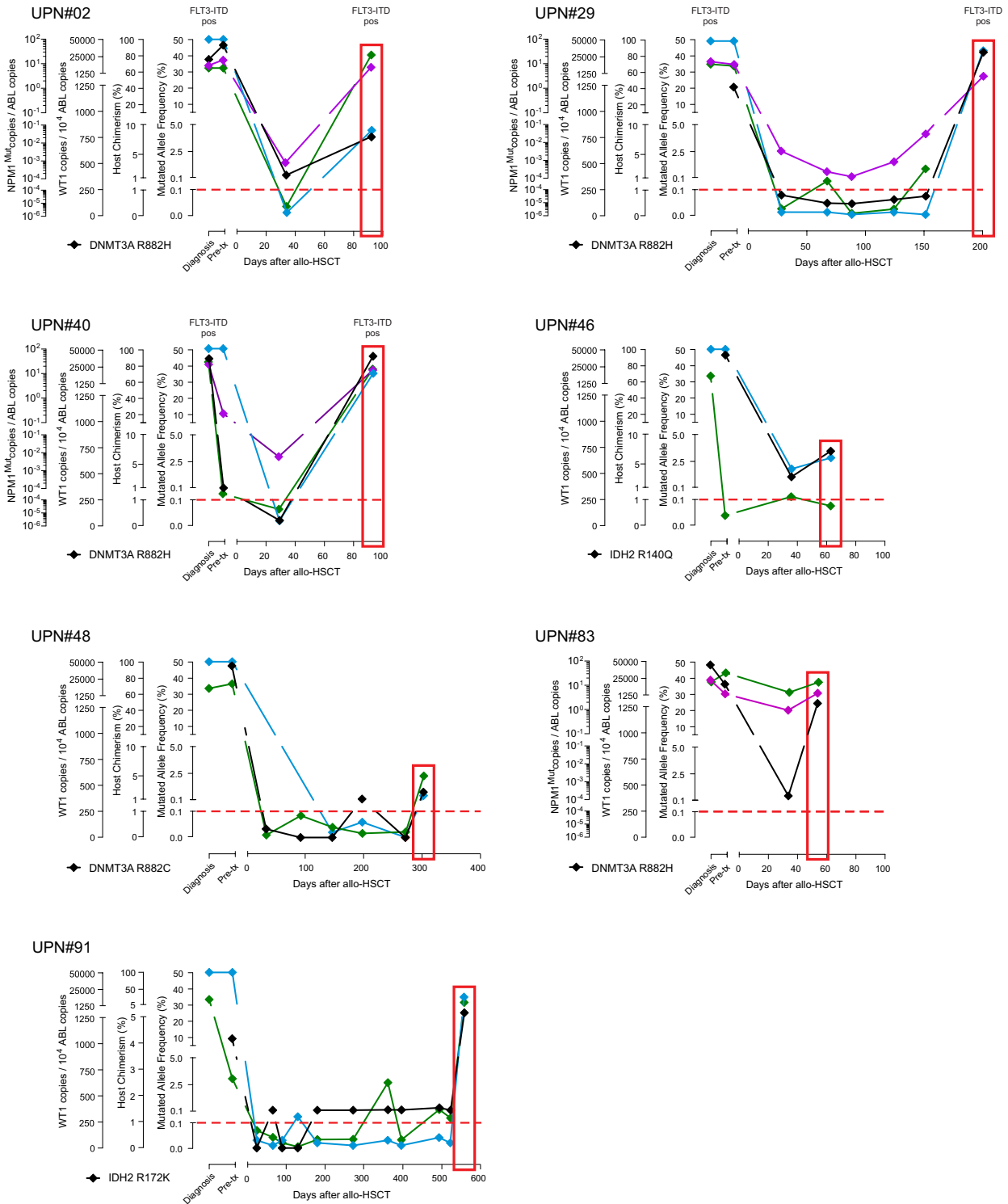
Supplementary Figure 1



Supplementary Figure 2

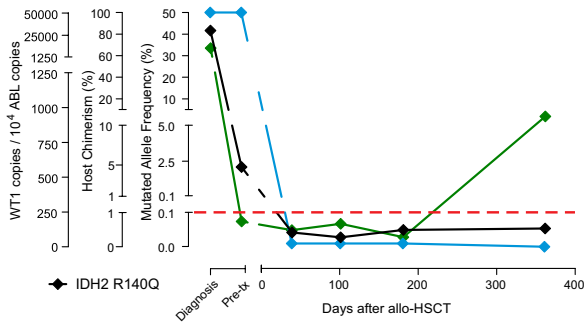


Supplementary Figure 3

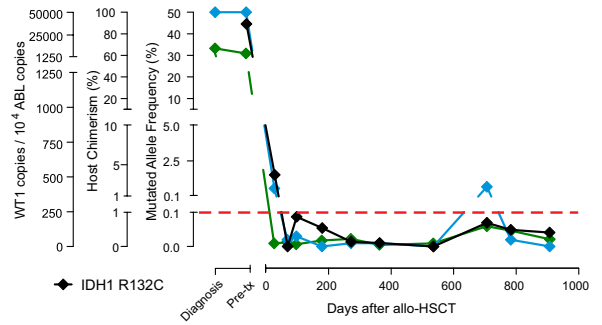


Supplementary Figure 4

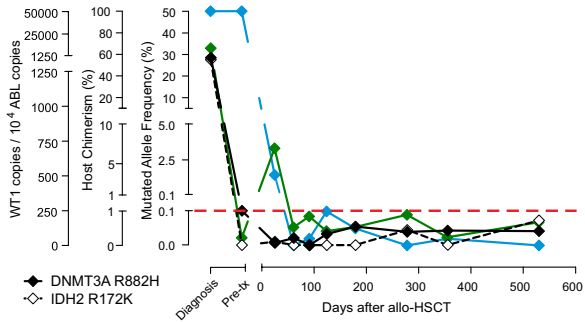
UPN#11



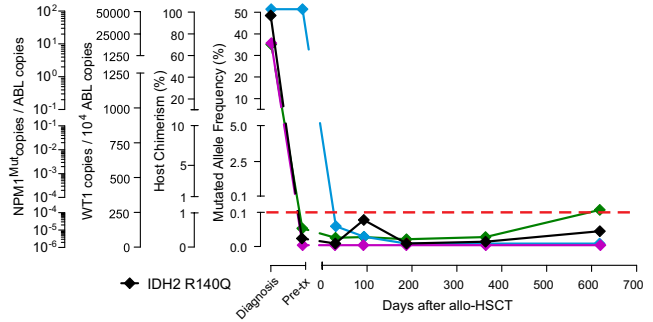
UPN#14



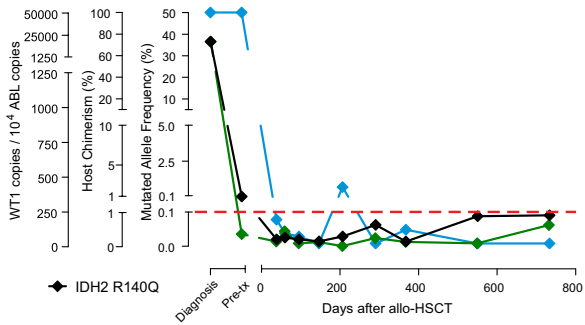
UPN#15



UPN#26



UPN#33



UPN#76

