

Supplementary Information

Biodistribution

Experimental Overview

The study was carried out using primary lineage negative (Lin-) murine bone marrow cells isolated from male B6.129S-*Cybb*^{tm1Din}/J mice (hereafter called **X-CGD mice**). Lin- cells were isolated according to standard protocols. Cells were transduced with **G1XCGD (lot H.11027.STD)** and transplanted into lethally irradiated female X-CGD mice. After hematopoietic reconstitution (6 weeks), organs (lung, liver, spleen, heart, brain, thymus, gonads, kidney, bone marrow and peripheral blood) of half of the treated animals was analyzed for the presence of *CYBB* sequences and engraftment by PCR (codon optimized *CYBB* cDNA and the SRY gene, respectively). In addition, cells from bone marrow, spleen and peripheral blood were analyzed by FACS for expression of *CYBB* (7D5 staining). The rest of the transplanted animals was observed for a total of 3 months and analyzed as described above.

GROUP	EXPERIMENTAL TREATMENT	Number	Gender
<u>A</u>	Test Group <ul style="list-style-type: none">G1XCGD transduced cells	12	F
<u>B</u>	Control Group <ul style="list-style-type: none">Non-transduced cells	2	F

Isolation of lineage negative bone marrow cells and transduction

Lineage negative (Lin-) BM cells were isolated and transduced as follows: Fresh Lin- cells isolated from complete BM of male X-CGD mice by magnetic sorting using lineage specific antibodies were prestimulated for 48 hours in STEMSPAN containing 100ng/ml mSCF, 100 ng/ml hTPO and 100 ng/ml hFLT3-L. Day 0 is defined as the day of isolation. Transduction was done in 24-well plates starting at day 2. Copy numbers were determined by qPCR after 10 days in culture. Vector copy numbers ranged between 0.51 and 1.0 per cell. Transgene expression was monitored by FACS after myeloid differentiation *in vitro* (10 to 14 days after transduction).

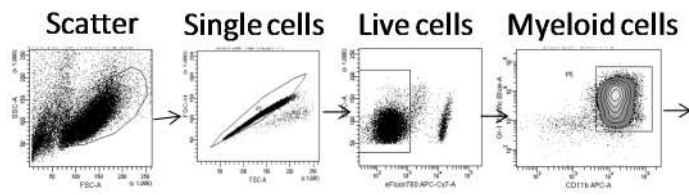
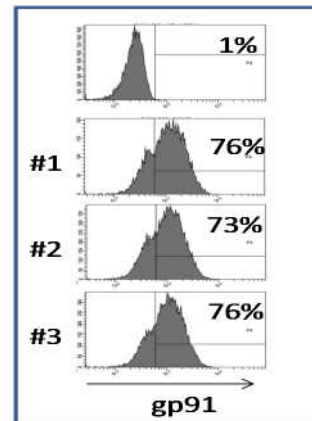
A**B**

Figure 1. Transduction efficiency in Lin⁻ cells. A. Gates used to identify myeloid cells. **B.** Intracellular staining for Gp91phox expression of X1CGD transduced cells pre-

Transplantation

Recipient mice (female X-CGD mice) were lethally irradiated using a split dose of 5.5 Gy each at days 3 and 4. Thereafter animals are reconstituted by tail-vein injection of 2×10^6 cells/animal.

Analysis

After hematological reconstitution (6 weeks) half of the animals were sacrificed and organs (lung, liver, spleen, heart, brain, thymus, gonads, kidney, bone marrow and peripheral blood) were analyzed for accumulation of X1CGD-transduced cells (short term effects). The rest of the animals was observed for a total of 12 weeks. Thereafter animals were analyzed as described above (long term effects)

Controls

Control animals transplanted with mock transduced cells were analyzed in parallel.

Time schedule

	Time							
Procedure	Day	Day	Day	Day	Day	Week	Week	Week
	0	2	4	10	14	5	6	12
BM collection from sygenic donor mice	X							
Transduction		X						
Vitality (Trypan Blue)	X	X	X					
Infusion of genetically modified cells			X					
Copy Nr				X			X	X
Engraftment (qPCR)							X	X
Gp91phox Expression by FACS					X	X	X	X
DHR activity						X		
Biodistribution analysis (short term)							X	
Biodistribution analysis (long term)								X

Cell Number and Viability

Samples were counted either with Trypan-Blue in conventional Neubauer-Chambers or the Scepter cell counter device from Millipore.

Determination of vector copy number

The mean vector copy number per cell (VCN) was determined by qPCR. Samples were measured in triplicates using 50 to 100 ng of genomic DNA. Primers and probe specific for the codon optimized gp91phox coding region and the human EpoR as endogenous standard were used to determine the amount of viral sequences per genome in a duplex reaction. A serial dilution of gDNA from a PLB985 clone harbouring a single lentiviral provirus was used for quantification of vector copy numbers.

Antibody-staining and flow cytometry

1×10^5 cells (from culture) or up to 1×10^6 cells (total BM) were resuspended in 200 μ l of FACS-buffer (1xPBS, 1mM EDTA, 0.5% BSA), antibodies and dead cell stain were added and the mix was incubated for 20min. Thereafter the FACS-tubes were filled to the top with staining buffer, pelleted at 300g for 5min at RT, the supernatant was discarded and the pellet resuspended for analysis on a FACS-Canto II device.

Colony assay and NBT-assay

The cells were counted and 2000 and 6000 cells were plated in Methocult H4433 (StemCell Technologies) in 3.5cm plates in duplicates according to the manufacturer's instructions. 14 to 16 days later an NBT-assay was performed to detect ROS-producing colonies. For this 750 μ l of freshly prepared NBT-solution (1mg/ml NBT in PBS + 2 μ g/ml PMA) was applied to each plate and incubated for 45min at 37°C. The reaction was stopped via transfer of the plates to 4°C. The colonies were counted immediately and scored for NBT+ve and NBT-ve. Only non-erythroid colonies were taken into account, as only those are capable of producing ROS.

Retronectin coating

Stock solutions of 1mg/ml Retronectin (Takara) were diluted in PBS to 10 μ g/cm². 400 μ l per 24-well in a non-tissue culture plate (BD Falcon) were applied and incubated for 2h at RT. Retronectin was removed, the wells were incubated with 1ml blocking

solution (PBS+ 2% BSA) for 30min RT, washed once with HBSS (Sigma) followed by PBS (PAA). Plates were stored with PBS until use (several hours).

Gp91phox expression by FACS

The product of the CYBB gene, a glycoprotein of 91kD (Gp91phox) localizes to internal membranes as well as to the cellular membrane and can be detected by the mouse anti-human monoclonal antibody 7D5 (MBL). The epitope recognized by the 7D5 antibody localizes to the extracellular peptide portion of primate gp91phox (Nakamura et al., 1987; Yamauchi et al., 2001).

DHR-assay

10^5 cells (*in vitro* cultured) or 10^6 (total BM) were pelleted at 300g for 5min at RT and resuspended in 200 μ l of room temperature reaction buffer (HBSS+Ca²⁺+Mg²⁺ supplemented with 7.5mM Glucose, 0.5% BSA, 5000U Catalase per ml) supplemented with Fc-block, antibodies, dead cell stain and DHR (Dihydrorhodamine123 at 1 μ g/ml). The suspension was transferred onto a 96well plate and incubated at 37°C under interval shaking (12s intervals). After 5min preincubation 10 μ l of a 5 μ g/ml PMA solution was added to start the reaction. 20min later the reaction was stopped by placing onto ice and recorded immediately on a FACS Canto II flow cytometer.

Human CYBB and β -actin qPCR validation

Primer/Probe sequences:

CYBB-cov (gp91) for: CCAGCAGCACCAAGACCAT

CYBB-cov (gp91)rev: CCGATGAAAAAGATCACGAACAG

Cov = codon optimized version

Probe: ACCAGAACACCTCGAAGTAGCTCCGCC (double-dye-FAM)

Target: bases 572-653 of the codon optimized gp91phox

Amplikon:

CCAGCAGCACCAAGACCATccGGCGGAGCTACTTCGAGGTGTTCTGGTacaccacac
cacCTGTTCGTGATCTTTTCATCGG 82bp

β-actin for: AGAGGGAAATCGTGCGTGAC

β-actin rev: CAATAGTGATGACCTGGCCGT

Probe: TexasRed-CACTGCCGCATCCTCTTCCTCCC-BHQ2

Target: chr5:143666074-143666211

Amplikon:

AGAGGGAAATCGTGCGTGACatcaaagagaagctgtgctatggtgctctagacttcgagcaggagatgg
cCACTGCCGCATCCTCTTCCTCCCtggagaagagctatgagctgcctgACGGCCAGGTCAT
CACTATTG 138bp

Instrument and cycling conditions:

Roche LightCycler480, FAM-filter set (Ex/Em): 483nm/533nm, TexasRed-filter set (Ex/Em): 558nm/633nm

5min 95°C activation and denaturation step

15s 95°C denaturation

} 45X

60s 60°C annealing, elongation and data acquisition

Each sample is run in triplicate.

Detection limit: classical definition: the amount at which 95% of the reactions give you a positive signal. Adjusted to our specific need. We evaluate what the minimum contribution of male DNA in a female background needs to be to reliably discriminate the specific signal from background signal.

Limit of quantification and linear range

To obtain informations about the limit of quantification and the linear performance range of our duplex qPCR serial dilutions of a gDNA from a Baf3-clone harboring a single proviral integration was used. The highest amount of template gDNA applied was 150ng, the lowest amount used was 0.69ng (Figure 1).

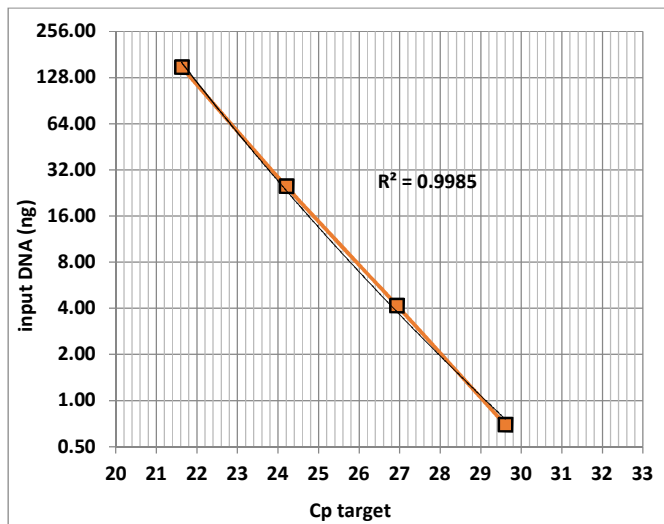


Figure 1.

A very high degree of linearity was observed over the whole test range. During the final experiments the average amount used for each qPCR-reaction was 50ng. The range tested here accordingly covers vector copy numbers in the range from 3 down to 0.013.

These results clearly indicate that the limit of detection (**LOD**) is **<0.013VCN or less than one proviral genome per 0.46ng gDNA.**

As the reaction showed a very high degree of linearity over the whole test range we conclude that this also equals the limit of quantification (**LOQ**): **>0.013VCN ore more than one proviral genome per 0.46ng gDNA.**

Repeatability and Reproducibility

Repeatability refers to short term interplate variations of the assay while repeatability includes long term interplate variations. Each 96-well plate used for sample analysis contained a full calibrator dilution series consisting of 150ng, 30ng, 6ng and 1.2ng

reference genomic DNA. Using the results from these internal controls we are able to monitor repeatability and reproducibility of this assay. A single plate was tested on week 0, three plates on week 6 and four plates on week 12 (Figure2).

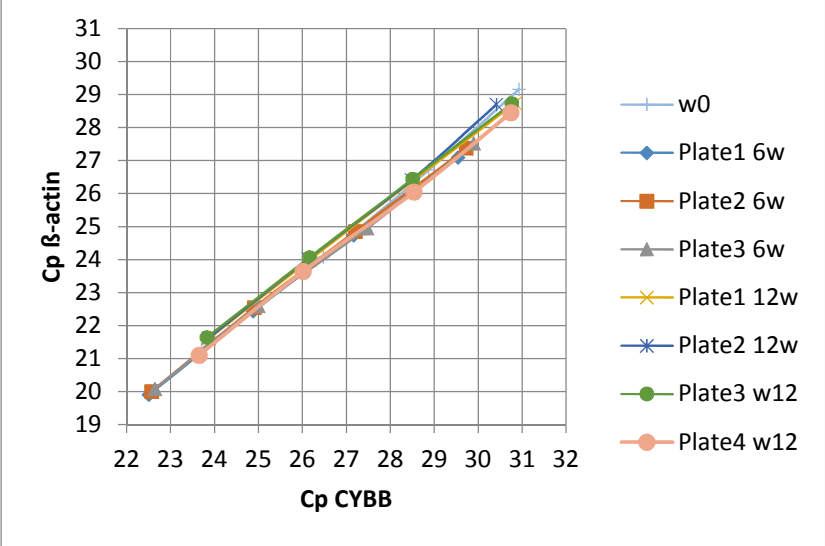
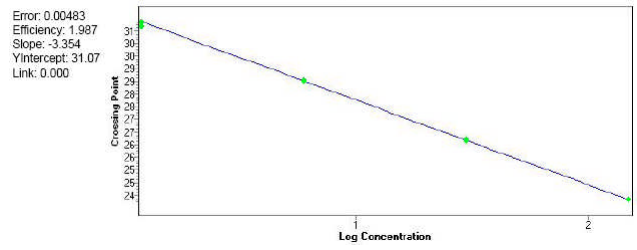
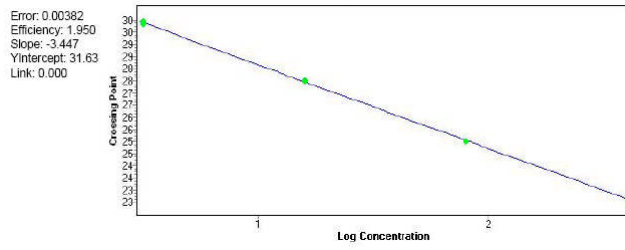
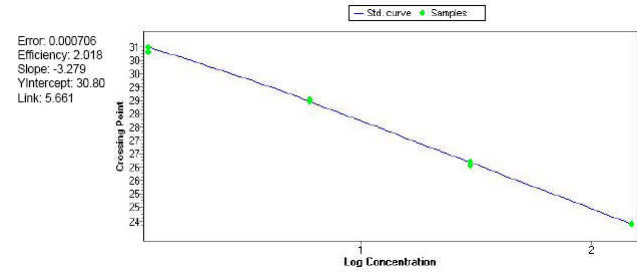
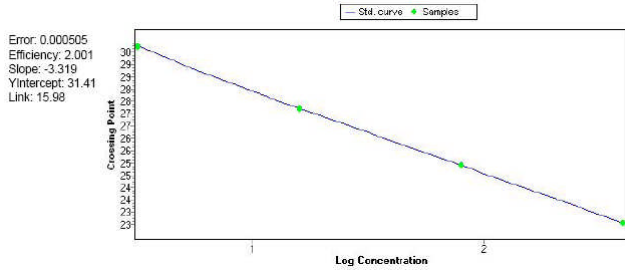
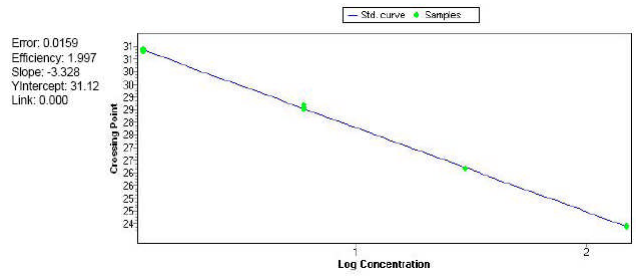
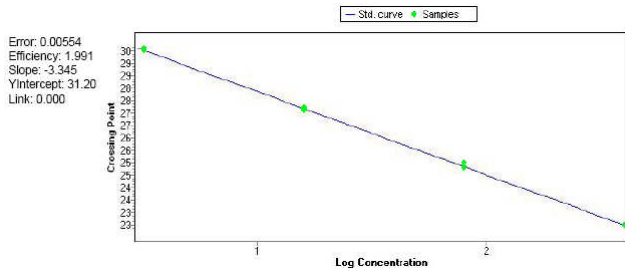


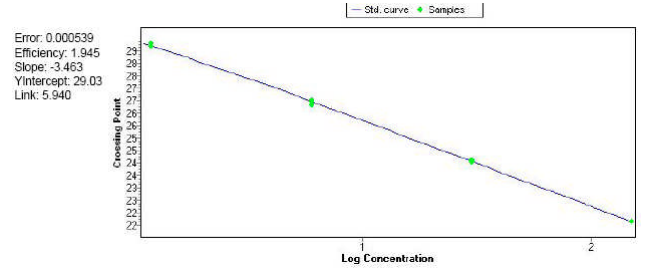
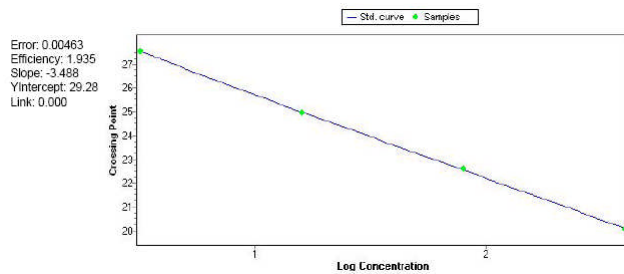
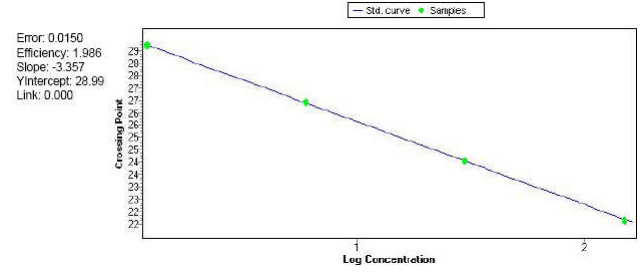
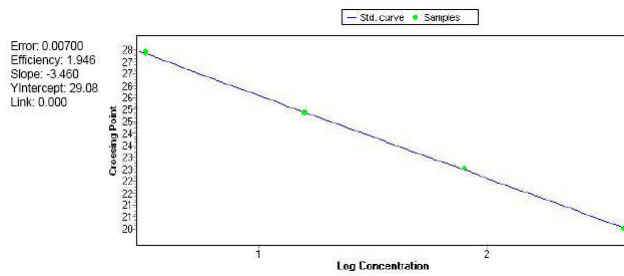
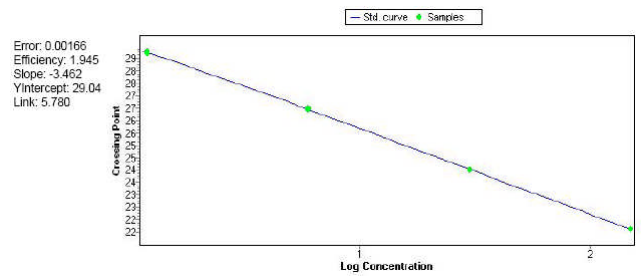
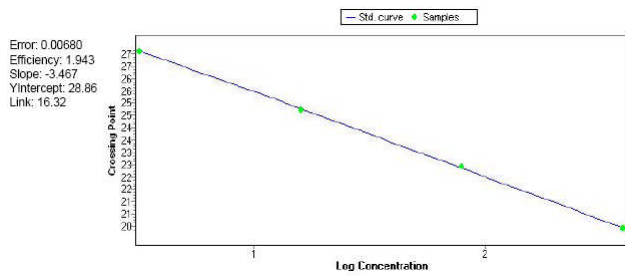
Figure 2.

The overlay of all Cp values clearly indicates a high degree of repeatability and reproducibility (Figure 2). Diagonal shifts stem from different amounts of gDNA applied for different repetitions of the reactions and further support the robustness of the reaction.

CYBB-cov (gp91) calibrator curves



m β -Actin calibrator curves



Reaction efficiency

From calibrator dilution series present on each plate we can calculate the reaction efficiency. The mean amplification efficiency of 7 separate experiments (Figure 4) was $99.2\% \pm 1.4\%$ for the SRY qPCR and $97.3\% \pm 1.02\%$ for the β -actin qPCR.

Analytical specificity of the reaction

To verify the specificity of the reaction we subjected different reaction-setups to agarose-gel analysis.

A B C



Lane A: duplex reaction with 150ng template after 45 cycles

Lane B: single reaction β -actin with 150ng template after 45 cycles

Lane C: single reaction CYBB-cov with 150ng template after 45 cycles

← 138bp (m β -actin)
← 82bp (CYBB-cov)

Even after 45cycles no significant unspecific amplicon was detected. The normal range of quantification is in the range between 20 to 30 cycles.

Quantification calibrator

According to MIQE Guidelines 7.3 the quantification calibrator should vary no more than 0.5 -1 Cp between experiments. The mean Cp change over time was 0.42 at maximum for CYBB-cov (mean 0.23 ± 0.14) and 0.54 for β -actin qPCR (mean 0.4 ± 0.11) during the total test period of 12weeks.

CYBB-cov									
6w				12w					
DNA / well	PCR1	PCR2	PCR3	DNA / well	PCR1	PCR2	PCR3	PCR4	
400	22.50	22.56	22.64	150	23.87	23.88	23.82	23.65	
80	24.88	24.90	24.99	30	26.17	26.14	26.16	26.01	
16	27.16	27.20	27.47	6	28.58	28.48	28.50	28.53	
3.2	29.54	29.72	29.89	1.2	30.83	30.41	30.76	30.74	
m β -actin									
6w				12w					
DNA / well	PCR1	PCR2	PCR3	DNA / well	PCR1	PCR2	PCR3	PCR4	
400	19.90	20.00	20.08	150	21.61	21.64	21.64	21.10	
80	22.43	22.54	22.59	30	24.00	24.03	24.06	23.64	
16	24.73	24.85	24.95	6	26.46	26.41	26.44	26.04	
3.2	27.09	27.37	27.52	1.2	28.74	28.70	28.73	28.45	

Table1

Summary and Conclusion:

The reaction parameters fulfill the criteria of the MIQE whitepaper, is highly reproducible and efficient. Using duplex qPCR minimizes pipetting errors while maintaining reaction specificity and linearity.

Murine SRY and β -actin qPCR validation

Primer/Probe sequences:

SRY for: agcctcatcggagggcta

SRY rev: aggcaactgcaggctgta

Probe: Roche mouse universal Probe #82 FAM-cagaggag-BHQ

Target: chrY:1919286-1919345

Amplikon:

AGCCTCATCGGAGGGCTAaagtgtcaCAGAGGAGtggcattTACAGCCTGCAGTTGCC

I 60bp

β -actin for: AGAGGGAAATCGTGCGTGAC

β -actin rev: CAATAGTGATGACCTGGCCGT

Probe: TexasRed-CACTGCCGCATCCTCTTCCTCCC-BHQ2

Target: chr5:143666074-143666211

Amplikon:

AGAGGGAAATCGTGCGTGACatcaaagagaagctgtgctatggtgctctagacttcgagcaggagatggcCACTGCCGCATCCTCTTCCTCCCtggagaagagctatgagctgcctgACGGCCAGGTCAT

CACTATTG 138bp

Instrument and cycling conditions:

Roche LightCycler480, FAM-filter set (Ex/Em): 483nm/533nm, TexasRed-filter set (Ex/Em): 558nm/633nm

5min 95°C activation and denaturation step

15s 95°C denaturation

} 45X

60s 60°C annealing, elongation and data acquisition

Each sample is run in triplicate.

Detection limit: classical definition: the amount at which 95% of the reactions give you a positive signal. Adjusted to our specific need:

We evaluate what the minimum contribution of male DNA in a female background needs to be to reliably discriminate the specific signal from background signal.

Thoughts: Generally the detection cutoff for engraftment is arbitrarily set to 0.1% in hematopoietic stem cell research.

Limit of quantification, linear range and comparison duplex vs split reactions

Three setups were tested to obtain the required information:

1. Serial dilution of male DNA (SRY target and β -actin internal control are equally diluted), duplex qPCR

Pro: This setup provides „Target“ and „Reference“ standard curve in the same series, minimizes pipetting errors, absolute template DNA quantification not required thus minimizing errors.

Con: Does not give you the detection limit of your „Target“ in an excess of genomic DNA.

This sample series was used as reference for the other setups because least error sources exist.

2. Serial dilution of male DNA while keeping total DNA-load constant by addition of female DNA, duplex qPCR

Pro: Mimicking the true situation in samples. Gives you the detection limit of the „Target“ sequence in a background of genomic DNA

Con: Prone to pipetting errors and errors in template DNA quantification.

3. Serial dilutions of male DNA, reactions for „Target“ and „Reference“ in separate wells, single qPCR

Pro: No interference between the PCR reactions or fluorescence spillover

Con: Highly prone to pipetting errors, double costs, half the sample load on one plate, sensitive to errors in template DNA quantification.

During the test run the lowest amount of male template DNA applied was 0.05ng in 200ng of female DNA which equals an engraftment of 0.025%. Until 0.2ng we observed a high degree of linearity and small deviations from linearity when using lower template amounts in SRY and β -actin qPCR reactions (Figure1). This is also reflected by the increasing error of triplicates only at the lowest template concentration of 0.05ng. Although deviations from linearity at these low template amounts are automatically taken into consideration during data analysis using the LC480 software, we decided to use 0.2ng as limit for quantification. This equals 0.5% male donor cells when using 40ng of template DNA (this is the lowest amount of sample DNA used in any experiment) and equals a Cp value of 32.9. Cp values higher than that are considered to be error prone.

LOQ: 0.2ng male DNA which correlates to $\leq 0.5\%$ engraftment

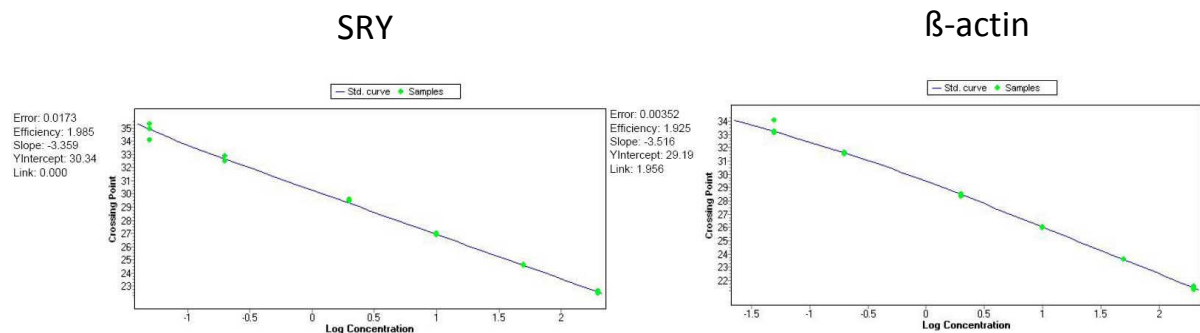


Figure1

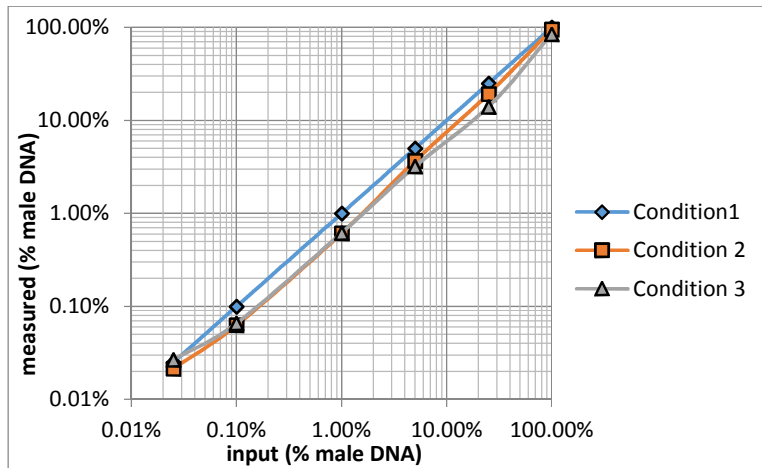


Figure2

Repeatability and Reproducibility

Repeatability refers to short term interplate variations of the assay while reproducibility includes long term interplate variations. Each 96-well plate used for sample analysis contained a full calibrator dilution series consisting of 150ng, 30ng, 6ng and 1.2ng male genomic DNA. Using the results from these internal controls we are able to monitor repeatability and reproducibility of this assay. A single plate was tested on week 0, three plates on week 6 and three plates on week 12 (Figure4). The overlay of all Cp values clearly indicates a high degree of repeatability and reproducibility (Figure3).

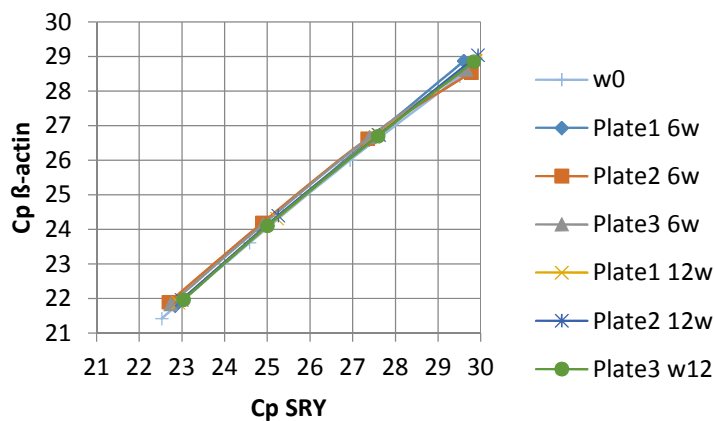
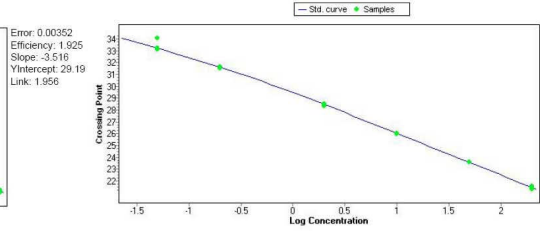
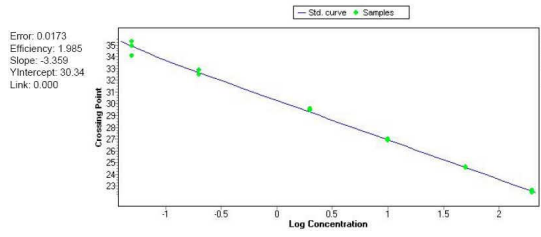


Figure3

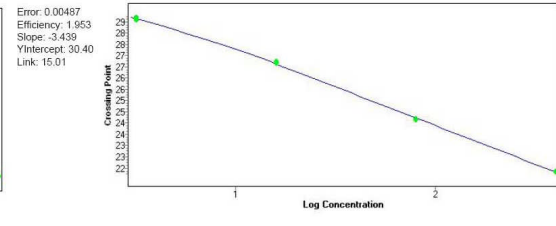
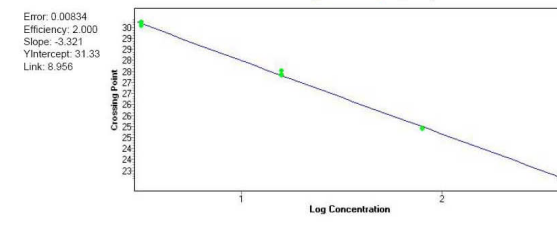
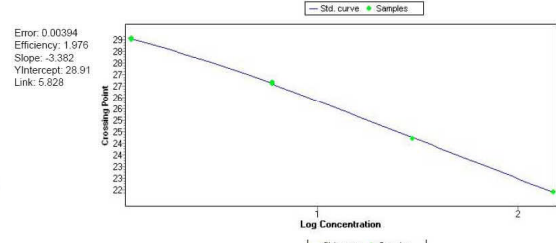
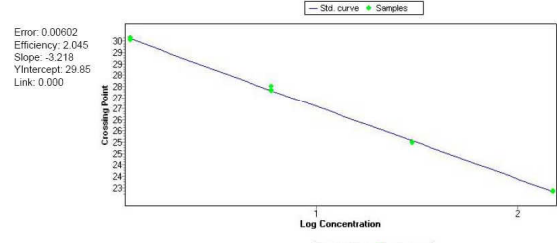
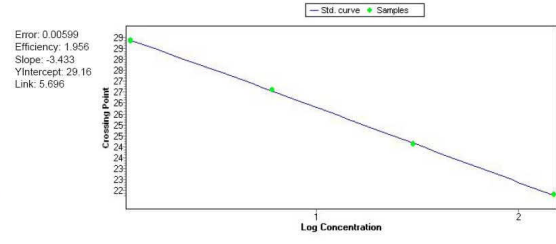
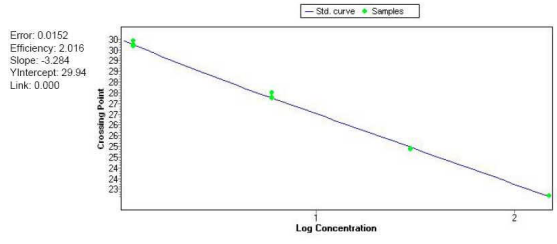
SRY 0w

beta-actin 0w



SRY 6w Plate1-3

β -actin 6w Plate1-3



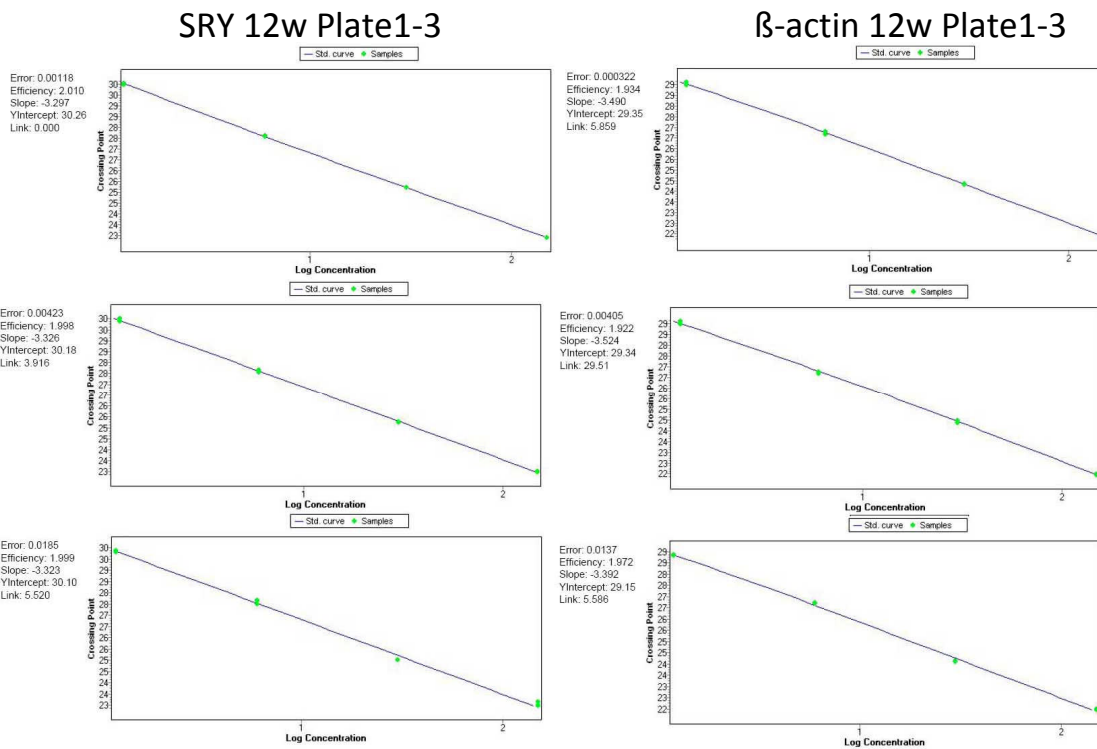


Figure4

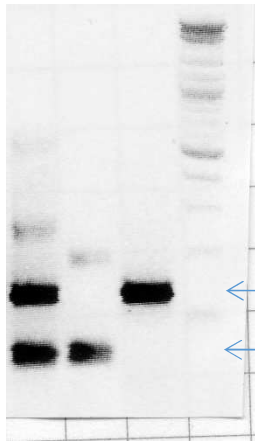
Reaction efficiency

From calibrator dilution series present on each plate we can calculate the reaction efficiency. The mean amplification efficiency of 7 separate experiments (Figure 4) was $100\% \pm 0.96\%$ for the SRY qPCR and $98\% \pm 1.09\%$ for the β -actin qPCR.

Analytical specificity of the reaction

This describes the specificity of the assay for the intended target sequence. Individual wells of the finished reaction (45cycles) were subjected to agarose gel analysis to check whether exclusively the expected PCR products are amplified.

1 2 3 Marker



1. Duplex reaction of a test sample where 100ng of template were applied.
2. SRY qPCR single reaction using the same template and conditions as in 1.
3. β -actin qPCR single reaction using the same template and conditions as in 1.

← β -act
← SRY

Control wells not containing male template DNA did not cross the threshold in the SRY qPCR during the total run of 45 cycles in 3 out of 4 triplicates in our test-run. Control cells without any template DNA did cross the threshold in individual wells in β -actin qPCR in the range of cycle 35 ± 1 .

Quantification calibrator

According to MIQUE Guidelines 7.3 the quantification calibrator should vary no more than $0.5 - 1$ Cp between experiments. The mean Cp change over time was 0.34 ± 0.06 at maximum for SRY and 0.28 ± 0.17 for β -actin qPCR during the total test period of 12weeks.

Table1

DNA / well	SRY Cp						β -act Cp					
	6w			12w			6w			12w		
	PCR1	PCR 2	PCR 3	PCR 1	PCR 2	PCR 3	PCR 1	PCR 2	PCR 3	PCR 1	PCR 2	PCR 3
150	22.84	22.69	22.73	22.91	22.99	23.03	21.79	21.89	21.84	21.88	21.96	21.96
30	25.00	24.88	24.90	25.22	25.25	25.00	24.10	24.19	24.15	24.32	24.40	24.10
6	27.39	27.34	27.39	27.59	27.61	27.59	26.60	26.62	26.67	26.74	26.73	26.70
1.2	29.60	29.77	29.65	30.01	29.93	29.83	28.87	28.54	28.62	29.05	29.04	28.85

Summary and Conclusion:

The reaction parameters fulfill the criteria of the MIQE whitepaper, is highly reproducible and efficient. Using duplex qPCR minimizes pipetting errors while maintaining reaction specificity and linearity. The maximum contribution of background signal observed in control samples in all performed assays translates to $\leq 1\%$ engraftment, but was $\leq 0.1\%$ or undetectable in the majority of cases.