Component-specific qPCR assays for characterization and identity testing of multigenome ZVex®, a dendritic cell-targeting lentiviral vector platform

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Background: ZVex is an integration-deficient, dendritic cell-targeting lentiviral vector platform currently being evaluated in clinical trials in sarcoma patients. We developed novel ZVex preparations encoding the full length human cancer testis antigens MAGE-A1, A3, A4, A10 as well as IL-12. Because lentiviral vectors package two RNA genomes each, this ZVex approach results in the generation of 15 possible permutations of homozygous or heterozygous vector genotypes. Here, we describe highly specific qPCR assays for detection of the 4 *MAGEA* genes and *IL12* for characterization and identity testing of ZVex preparations.

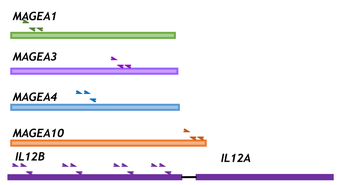
Component-specific qPCR assays for ZVex encoding five genes

Table 1: % Sequence identity between the *MAGEAs*

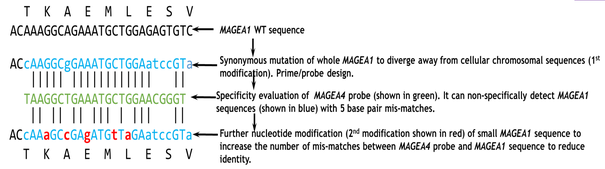
	MAGEA1	*MAGEA3*	*MAGEA4*	*MAGEA10*
MAGEA1	-	80.9	87.3	66.0
MAGEA3	70.6	-	83.0	67.3
MAGEA4	87.3	72.7	-	67.5
MAGEA10	65.8	54.6	67.6	-

Tools: LALIGN (X. Huang and W. Miller, 1991, Adv. Appl. Math. 12:373-381)
Nph Align (Pearson, W.R., Wood, T., Zhang, Z., and Miller, W. (1997)

B Location of component-specific assays



A *MAGEA* gene design



The homologous nature of the *MAGEA* family members (~50-90% sequence identity) poses significant challenges to their specific detection and quantification via qPCR in a *MAGEA* gene pool (**Table 1**). *MAGEA1*/*IL12* genes were codon-modified (*MAGEA1* gene shown as an example, **Figure A, 1st modification)** to enable specific detection of vector expressed genes over cellular genomic sequences. For *IL12* detection the entire *IL12B* subunit was used as a template to design 4 separate assays from which the 4th amplicon was down-selected based on qPCR performance. For the 4 *MAGEA* genes, short, non-overlapping, divergent target regions in each of the genes were carefully selected as templates for primer/probe design (**Figure B**). To further enhance the specificity of the primers/probes to each of their target *MAGEA*, potential off target amplicons that can be generated in the other 3 *MAGEA* genes due to base pair mismatch were further sequence-modified to reduce sequence identities (non-specific binding of *MAGEA4* probe to *MAGEA1* sequences with 5 base pair mis-matches and further sequence modification of *MAGEA1* gene to reduce non-specific detection via *MAGEA4* probe is shown as an example in **Figure A.)**

Individual *MAGEA*-specific assays preserve quantitative accuracy in a complex sample matrix containing RNA from other *MAGEA* vector genomes

Table 4: *MAGEA* genome quantification in the presence of other *MAGEAs*

Target/Assay	Genomes/ml: *MAGEA* specific RT-qPCR with water as sample matrix	Genomes/ml: *MAGEA* specific RT-qPCR with *MAGEA* mix (A1,A3,A4,A10) sample matrix
MAGEA1	7.1e10	7.7e10
MAGEA3	1.2e11	1.2e11
MAGEA4	2.3e10	2.6e10
MAGEA10	4.7e10	5e10

Individual *MAGEA* vector RNA was spiked at known quantities into a water matrix or a matrix consisting of RNA extracted from the other three *MAGEA* vector preparations. The RNA was reverse transcribed and amplified using individual *MAGEA*-specific assays described in **Figure C-E.** The concentration of experimental samples was determined by extrapolation from a DNA standard curve based on Cq values and results are reported as genomes/ml. *MAGEA* specific assays generate similar genome titers in water and mixed *MAGEA* matrices indicating that they can be used to accurately quantify respective target genomes in the presence of the other *MAGEA* vectors (**Table 4**) and are amenable to multiplexing. Similar experiments were performed to confirm the same for the *IL12* assay (data not shown).

Component-specific assays specifically detect reverse transcribed DNA in a cell-based transduction assay over cellular genomic sequences

Table 5: *MAGEA*/*IL12* infectious units quantification in a cell-based transduction assay

	Infectious units/ml				
	MAGEA1	*MAGEA3*	*MAGEA4*	*MAGEA10*	*IL12*
MAGEA-transduced cell lysate	2.1e10	4.8e9	6.6e9	4.6e9	1.9e9
Mock cell lysate	No signal	No signal	No signal	No signal	No signal

Cells permissive to ZVex transduction were transduced using similar genomes of single gene vector preparations. After 16 hours, cells were lysed and assessed for *MAGEA* DNA using assays specific for each of the four *MAGEA* vector genomes. qPCRs were performed using the SSO advanced supermix or the iQ Powermix. The concentration of experimental samples was determined by extrapolation from DNA standard curves based on their Cq values and is reported as infectious units/ml. Component-specific assays specifically detect reverse-transcribed vector DNA with no detectable background signal from cellular chromosomal sequences(**Table 5**) indicating that the vector gene design with synonymous mutations was successful in achieving sufficient sequence divergence (1st modification in **Figure A**).

Component-specific vector titer values correlate well with transfected plasmid ratios in singleplex assays

F Five-genome ZVex composition made by transfecting 0.5 mg of *IL12* plasmid and 0.125 mg each of the *MAGEA* plasmids

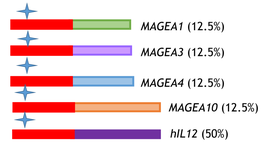


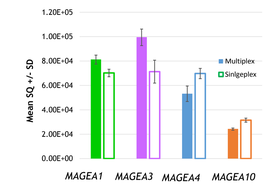
Table 6: Component-specific genome titers for ZVex using the singleplex assay

qPCR Assays	Genomes/ml (%CV, n>=3)	Expected % of the transgene	Observed % of the transgene
Gold standard (Total)	1.6e11 (29%)		
MAGEA1	3.4e10 (31%)	12.5	21.3
MAGEA3	9.3e9 (29%)	12.5	5.7
MAGEA4	2.7e10 (25%)	12.5	16.8
MAGEA10	2e10 (27%)	12.5	12.3
hIL12	8.6e10 (35%)	50	53.2

ZVex vector preparations were generated by transfecting producer cells with a mix of 5 different plasmids (50% *IL12* and 12.5% of each of the 4 *MAGEA* plasmids) encoding the codon modified full length *MAGEA* and *IL12* genes along with essential vector packaging plasmids (**F**). Following harvest and downstream purification, vector RNA was extracted and quantified using the assays described above. Component-specific assay primer and probe reagents were used in conjunction with a "gold standard" primer/probe that detects an amplicon common to all the plasmids (as denoted by a ✦) and thus measures total vector genome titers. The concentration of experimental samples was determined by extrapolation from a DNA standard curve based on their Cq values and is reported as genomes/ml. Relative genome-specific titers were computed by dividing component specific titers by total genome titers and reported as % of total vector titers. Individual component specific assays correlate well with expected titers based on relative transfected plasmids amount with *IL12* producing the highest titer and the 4 *MAGEA*s producing similar lower titers (**Table 6**). The titers are within the 2-3 fold variability observed in qPCR assays and hence do not exactly match either the expected percentage of the transgene or add up to 100%.

Pilot multiplexing of *MAGEA RT*-qPCRs

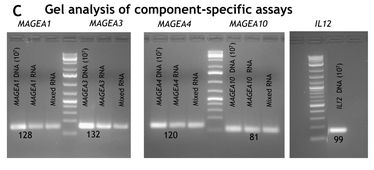
G Mean starting quantity for single *MAGEA* vector RNA run in single and multiplex qPCR reactions



RNA was extracted out of single ZVex vectors individually containing each of the 4 *MAGEA* genes. For multiplexing, same gene specific probe sequences as used in singleplex assays were tagged with different fluorophores as specified: *MAGEA1*-FAM, *MAGEA3*-HEX, *MAGEA4*-Texas Red, *MAGEA10*-Cy5 . Singleplex and multiplex RT-qPCRs were performed using iTaq 1-step Universal probes kit. The single and multiplex assays measured comparable RNA quantities indicating that the *MAGEA* assays can detect their respective target genes in the presence of each other and are amenable to multiplexing. The error bars represent standard deviation of 3 qPCR replicates.

Specificity, qPCR efficiency, quantitative ability of component-specific assays

C Gel analysis of component-specific assays



D Melt curve analysis of component-specific assays

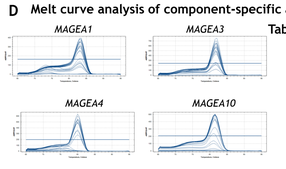


Table 2: Specificity of *MAGEA* assays on single and mixed RNA

Target	*MAGEA* assays			
	M1	M3	M4	M10
MAGEA1	+	-	-	-
MAGEA3	-	+	-	-
MAGEA4	-	-	+	-
MAGEA10	-	-	-	+
All 4 MAGEAs	+	+	+	+

+ Can detect >= 10 copies/reaction
- Cannot detect 10^7 copies/reaction

E qPCR efficiency of *MAGEA1* and *IL12* assays

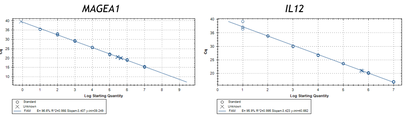


Table 3: Quantitative ability of component-specific and a validated "gold standard" RT-qPCR

Target/Assay	Genomes/ml: Component-specific RT-qPCR assay (%CV, n >=3)	Genomes/ml: Gold standard RT-qPCR assay (%CV, n>=3)
MAGEA1	3.6e11 (2.4%)	2.8e11 (0.5%)
MAGEA3	5.2e10 (5.4%)	5.6e10 (3.5%)
MAGEA4	1.5e10 (5.3%)	9.9e9 (10.8%)
MAGEA10	1.7e10 (6.2%)	6e10 (6.8%)
IL12	1.9e11 (6.3%)	2.6e11 (3.3%)

The *MAGEA*/*IL12* primer/probes were used to amplify 10^7 copies of *MAGEA*/*IL12* DNA or RNA extracted out of single genome or multigenome vectors. RT-qPCR reactions were performed using the using RNA UltraSense One-Step Quantitative RT-PCR kit. *MAGEA* assays specifically amplified their target sequences in the absence and presence of other 3 *MAGEAs* (**Table 2**) as shown by the presence of a single qPCR product at the expected size (**C**) and a presence of a single peak during melt curve analysis (**D**) (also observed for *IL12*, data not shown). The primer/probes specifically detected from 10 to 10^7 copies of their target genes. An 8 point DNA standard curve obtained with *MAGEA1* and *IL12* assays is shown as an example in **Figure E.** Individual component-specific assays benchmark well against a "gold standard" RT-qPCR assay (as shown in **Figure F**) used for quantifying total vector genomes (**Table 3**).

Component-specific titers correlate well with transfected plasmid ratios in multiplex assays

H Five-genome ZVex composition made by transfecting 0.5 mg of *GFP or IL12* plasmid and 0.125 mg each of the *MAGEA* plasmids

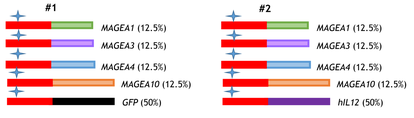


Table 7: Component-specific genome titers for ZVex using the multiplex assay

		Total/IL-12 duplex	Multiple *MAGEA* genomes 4-plex				
		Genomes/ml					
Vector	Gold standard (Total)	*IL12*	*MAGEA1*	*MAGEA3*	*MAGEA4*	*MAGEA10*
#1	9.8E10	<LOD	1.3E10	1.5E10	7.4E9	7.6E9
	Observed %	**<LOD**	**13%**	**15%**	**8%**	**8%**
#2	1.3E11	4.8E10	1.8E10	8.9E9	1.1E10	9.8E9
	Observed %	**36%**	**13%**	**7%**	**8%**	**7%**
Expected %:		50%	12.5%	12.5%	12.5%	12.5%

The multiplex assay was made more sensitive and quantitative by replacing the single step RT-qPCR reaction with a 2-step RT-qPCR reaction, consisting of separate cDNA synthesis and cDNA amplification steps. Since the qPCR machine can only detect 4 fluorophores at a time, the multiplex assays were split into two: 1) A duplex assay consisting of *IL12* and a "gold standard" assay used to quantify total vector genome titers with FAM and Texas-Red as the respective probe fluorophores, and 2) a 4-plex assay consisting of the 4 *MAGEA* primer/probes with FAM, HEX, Texas-Red, Cy5 as the fluorophores for the respective *MAGEA* probes. Two sets of ZVex vector preparations were produced as described previously by transfecting 5 different plasmids (either 50% *GFP* or *IL12* and 12.5% of each of the 4 *MAGEA* plasmids) (**H**). cDNA synthesis was performed using extracted vector RNA, gene specific reverse primers, and iScript Select cDNA synthesis kit. cDNAs were amplified using the iQ Multiplex Powermix. Individual vector genomes titers were calculated as described above using a universal standard consisting of a synthetic DNA gene containing all the 6 amplicons. Percentages denote the relative amount of each transgene present in the vector preparation shown as the percentage of total vector genomes. **Table 7** shows that the observed transgene concentrations agree with the expected values indicating that multiplex assays accurately measure their target genes in ZVex preparations.

Conclusions

- Component-specific RT-qPCR and qPCR assays were developed for the specific assessment of sequence modified genes in ZVex preparations encoding full length MAGE-A1, A3, A4, A10 and IL-12.
- The assays demonstrate relative gene-specific titer values that correlate well with transfected plasmid ratios. *IL12* and individual *MAGEA*-specific qPCR assays benchmark well against previously established "gold standard" assays currently used for quantifying total ZVex vector genome titers.
- These assays demonstrate inter- and intra-gene specificity, can be used in a single or multiplex format and can be used for titration, characterization, and identity release testing of ZVex lots produced to deliver multiple vector genomes.