Development and validation of a multiplex qPCR assay for RCL monitoring

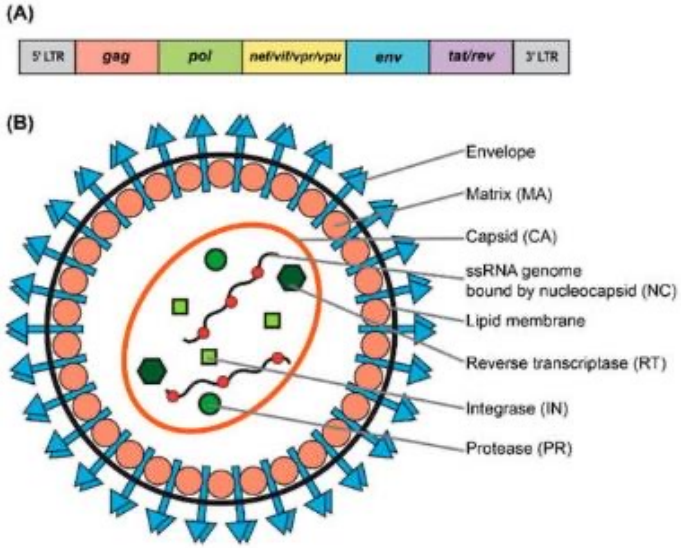
Lara Duchstein | EBF Open Symposium | 16Nov2023

BioAgilytix

Cell Therapy

Replication Competent Lentivirus (RCL)

- Lentiviral vectors are commonly used to introduce new and corrected genes into cell therapy products for treatment of human diseases.
- Lentiviral vectors are ideal for delivery and stable integration of genes of interest into the host cell genome.
- However, they potentially pose risks to human health, such as integration-mediated transformation and generation of a replication competent lentivirus (RCL) capable of infecting non-target cells.
- FDA requirement: RCL monitoring



Dong, W.; Kantor, B. Lentiviral Vectors for Delivery of Gene-Editing Systems Based on CRISPR/Cas: Current State and Perspectives. Viruses ***2021****, 13, 1288.*

Method Development Strategy

RCL assay

- fast
- cost-effective
- easy to handle
- **sensitive (50 copies/µg gDNA)**

qPCR RCL assay

- Primers and probe universally applicable and independent of the introduced gene of the respective cell therapy program
  → **envelope gene (VSV-G) sequence as target**
- Negative results need to be reliable
  → **analysis of endogenous reference gene (human ALB) in the same reaction**

Multiplex qPCR RCL assay

- Generation of RCL most unlikely event → tested samples expected to be negative
  → **Focus for Development and Validation on sensitivity**
- No cross-reactivity between primers and probes
  → **high specificity**

Assay Workflow

PBMCs

MagMAX™ DNA Multi-Sample Ultra 2.0 Kit

- Extraction of genomic DNA

DNA

- Quantification of genomic DNA
- Dilution to 20 ng/µl

DNA

- qPCR (duplex reaction) running in triplicates for 45 cycles in total

Quantitative Results

Pictures from Thermo Fisher Scientific website

Development Results

Specificity

- **dsDNA fragments containing either the VSV-G sequence or the ALB sequence only spiked at MQC spike level (2,500 copies/well)**
- **Acceptance Criteria**
  - VSV-G reagents must detect the VSV-G template and must show undetectable signal with ALB template
  - ALB reagents must detect the ALB template and must show undetectable signal with VSV-G template

COPIES/WELL						
	VSV-G MQC (2,500)			ALB MQC (2,500)		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
VSV-G SPx	2,446	2,529	2,494	Undetermined	Undetermined	Undetermined
ALB SPx	Undetermined	Undetermined	Undetermined	2,046	2,095	2,149

RCL Assay Set-up

Standards and Controls

- **Reference Material** = dsDNA fragments containing VSV-G or ALB sequence
- **Standard Curve** = VSV-G and ALB spiked in surrogate matrix (100 ng salmon sperm DNA per reaction)
- STD1 = 1,000,000 copies/well STD8 = 5 copies/well
- **QCs** = VSV-G and ALB spiked in PBMC pool DNA (100 ng/rxn)
- **NQC** = unspiked PBMC pool DNA freshly prepared for each run
- **NTC** = unspiked surrogate matrix

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 1	Standard 1	Standard 1	HQC_1	HQC_1	HQC_1						
B	Standard 2	Standard 2	Standard 2	MQC_1	MQC_1	MQC_1						
C	Standard 3	Standard 3	Standard 3	LQC_1	LQC_1	LQC_1						
D	Standard 4	Standard 4	Standard 4	NQC_1	NQC_1	NQC_1				HQC_2	HQC_2	HQC_2
E	Standard 5	Standard 5	Standard 5	NTC_1	NTC_1	NTC_1				MQC_2	MQC_2	MQC_2
F	Standard 6	Standard 6	Standard 6							LQC_2	LQC_2	LQC_2
G	Standard 7	Standard 7	Standard 7							NQC_2	NQC_2	NQC_2
H	Standard 8	Standard 8	Standard 8							NTC_2	NTC_2	NTC_2

Method Validation Strategy

Parameters

- **COU:** to assess the presence of the envelope gene (VSV-G) sequence for the purpose of Replication Competent Lentivirus (RCL) monitoring using DNA isolated from PBMCs

- **Full assessment for target gene (VSV-G)**
  - Calibration Curve Range and Linearity
  - Precision
  - Accuracy
  - Selectivity
  - Specificity
  - Sensitivity (LoB / LoD)
  - Dilutional Linearity
  - Sample / DNA Stability

- **Reduced assessment for reference gene (ALB)**
  - Calibration Curve Range and Linearity
  - Precision → Dilutional Linearity
  - Accuracy → Analysis of unspiked single donor samples for variation of target concentration (100 ng/rxn = 30,000 copies/rxn)
  - Selectivity → only unspiked single donor samples
  - Specificity
  - Sensitivity (LoB only)
  - Sample / DNA Stability

Method Validation Strategy

Acceptance Criteria

- **Based on *Best Practices in qPCR and dPCR Validation in Regulated Bioanalytical Laboratories***

	Standard Curve	QCs and validation samples
VSV-G	**%CV for Ct values** ≤ 2% for STD 1-5 ≤ 3% for STD 6-8	**%CV and %RE for BCC** ≤ 25% and ± 25% for ULOQ/HQC/MQC ≤ 45% and ± 45% for LQC/LLOQ1/2
ALB		**%CV for BCC** ≤ 25%

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Meeting Report

Best Practices in qPCR and dPCR Validation in Regulated Bioanalytical Laboratories

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INTRODUCTION

Cellular and gene therapies represent a new wave of rational therapeutic design by genetically modifying cells to carry out therapeutic tasks. While gene therapies use nucleic acids, cell therapies involve use of cellular material to replace missing functional proteins or alter cellular networks responsible for a diseased state. An increased understanding of disease-associated biological pathways and our ability to genetically encode cell and gene therapeutic programs have made treating monogenic disorders and hematological malignancies possible over the past two decades [1].

BACKGROUND

Examples of these novel drug modalities are the chimeric antigen receptor (CAR) cell therapies that utilize an *ex vivo* reprogramming of immune cells of cancer patients by inserting a gene for CAR; these cells are then transplanted back into the patient for effective targeting and killing of tumor cells [2]. Similarly, an *ex vivo* viral transduction of the functional β-globin locus into $CD34^+$ hematopoietic stem and progenitor cells has demonstrated remarkable clinical success in treating β-thalassemia patients [3]. Gene-editing tools based on zinc-finger nucleases and transcription activator-like effector nucleases (TALEN) have also surfaced for treatment of patients infected with HIV [4]. The recent advances in CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) systems are generating encouraging results in animal models [5] and will continue to propel the field of cell and gene therapy for development of novel treatment options for previously intractable diseases.

Although cell and gene therapies are demonstrating promising clinical results, an important limitation of many current approaches is that they provide little control over the dosage and cellular context of the therapeutic effect [6]. For example, clinical trials using CAR-T (chimeric antigen receptor T cells) have reported several fatal or life-threatening adverse events, including cytokine release syndrome and neurotoxicity related to excessive activation of engineered T cells [7].

There is a critical need for method development, validation and life cycle management of molecular methods, specifically quantitative PCR (qPCR) and digital PCR (dPCR) or NGS (Next Generation Sequencing), which are being used to support bioanalytical workflows for persistence monitoring, biodistribution, viral shedding, copy number and gene expression changes to support effective and reproducible therapies in this area. While emerging NGS promises to monitor both the efficacy of editing, as well as potential off-target gene edits with high sensitivity, the technology requires extensive infrastructure and is challenging to validate. Both qPCR and dPCR have currently emerged as a method of choice for bioanalytical assays and have their own pros and cons that are described in the qPCR platform comparison section and illustrated in Table I.

The emergence of novel cell and gene therapies combined with the evolution of molecular platforms that can be used to support drug development studies have made it imperative to understand the best practices and recommendations for developing and validating qPCR assays that could be used in regulatory submissions. Recently, global regulatory agencies including US Food and Drug Administration (FDA) have developed guidelines for monitoring and assessment of nonclinical and clinical studies to avoid adverse events [8]. These agencies have recommended and provided framework for use of molecular assays to assess long-term efficacy and safety outcomes. However, these guidelines do not address the bioanalytical requirements for characterizing and properly validating qPCR and dPCR assays. Thus, relying on the combined experience of contract research organizations, this paper outlines the recommendations for qPCR/dPCR assay design, method development, assay validation, acceptance criteria and subsequent sample analysis as recently discussed

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Standard Curve

Set-up / Acceptance Criteria / Performance

- **Standard Curve** = VSV-G and ALB spiked in surrogate matrix (100 ng salmon sperm DNA per reaction)
- **Reference Material** = dsDNA fragments containing VSV-G or ALB sequence

Standard Level	Nominal Concentration of VSV-G and ALB (copies/well)	Target Plate Acceptance Criteria	Validation Result
STD1	1,000,000	Triplicate well CV for Ct values ≤ 2%	Passed
STD2	100,000		Passed
STD3	10,000		Passed
STD4	1,000		Passed
STD5	100		Passed
STD6	50	Triplicate well CV for Ct values ≤ 3%	Passed
STD7 (LLOQ1)	10		Passed in 100% for VSV-G Passed in 96% for ALB
STD8 (LLOQ2)	5		Passed in 73% for VSV-G Passed in 73% for ALB
Overall	n/a	$R^2 \geq 0.980$ Efficiency: 90-110%	Passed

Validation Results

Precision and Accuracy – target gene

- **ULOQ (1,000,000) / HQC / MQC / LQC / LLOQ1 (10) / LLOQ2 (5 copies/well)**
- **Acceptance Criteria**
  - ULOQ / HQC / MQC: %CV ≤ 25% and ± 25% RE
  - LQC / LLOQ1 / LLOQ2: %CV ≤ 45% and ± 45% RE

INTRA-ASSAY MEAN (COPIES/WELL)

	ULOQ (1,000,000)		HQC (50,000)		MQC (2,500)		LQC (30)		LLOQ1 (10)		LLOQ2 (5)	
copies/well	1,167,351		54,570		2,781		30.9		8.87		2.40	
	%RE	**%CV**	**%RE**	**%CV**	**%RE**	**%CV**	**%RE**	**%CV**	**%RE**	**%CV**	**%RE**	**%CV**
	16.7%	1.4%	9.1%	0.5%	11.2%	1.6%	3.0%	13.1%	-11.3%	26.5%	-52.0%	45.7%

INTER-ASSAY MEAN (COPIES/WELL)

	ULOQ (1,000,000)		HQC (50,000)		MQC (2,500)		LQC (30)		LLOQ1 (10)		LLOQ2 (5)	
copies/well	1,066,831		53,777		2,867		35.3		10.7		4.06	
	%RE	**%CV**	**%RE**	**%CV**	**%RE**	**%CV**	**%RE**	**%CV**	**%RE**	**%CV**	**%RE**	**%CV**
	6.7%	6.7%	7.6%	1.5%	14.7%	3.2%	17.7%	19.9%	6.7%	33.7%	-18.8%	36.7%

** Intra-assay data exemplary shown for 1 run (out of 6)*

Sensitivity in qPCR assays

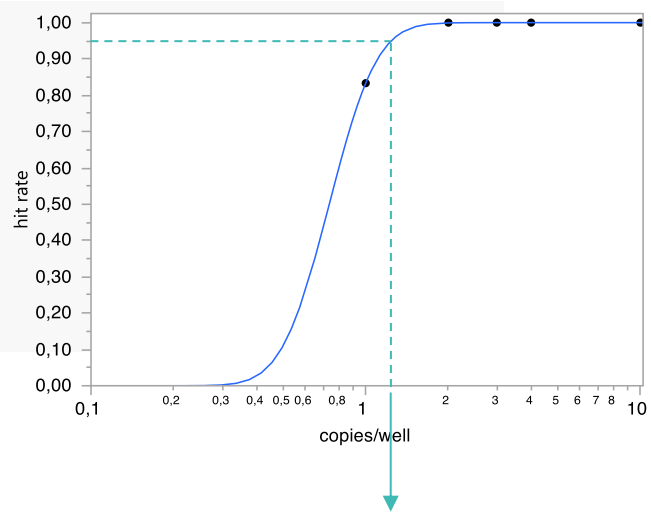
LLOQ / LOD / LOB

- **LLOQ = Limit of Quantification**
  passed acceptance criteria in terms of accuracy and precision
  general FDA requirement: LLOQ = 50 copies/µg DNA → 5 copies/well (100 ng total DNA)
- **LOB = Limit of Blank**
  background noise in negative samples
  mean Ct value - 1.645*SD
  → All LOB replicates Undetermined → LOB = Ct (45)
- **LOD = Limit of Detection**
  lowest spike concentration at which 95% of positive samples give a positive result (Ct < LOB) without the need to be quantified as an exact value (no target acceptance criteria in terms of accuracy and precision)

Validation Results

Sensitivity

Run ID	Set	LLOQ1 10 copies/well	LOD4 4 copies/well	LOD3 3 copies/well	LOD2 2 copies/well	LOD1 1 copies/well
1	1	pos	pos	pos	pos	pos
	2	pos	pos	pos	pos	pos
2	1	pos	pos	pos	pos	pos
	2	pos	pos	pos	pos	neg
3	1	pos	pos	pos	pos	pos
	2	pos	pos	pos	pos	neg
4	1	pos	pos	pos	pos	pos
	2	pos	pos	pos	pos	pos
5	1	pos	pos	pos	pos	pos
	2	pos	pos	pos	pos	pos
6	1	pos	pos	pos	pos	pos
	2	pos	pos	pos	pos	pos
Observed number of hits		12	12	12	12	10
Expected number of hits		12	12	12	12	12
Hit Rate (observed/expected hits)		1	1	1	1	0.833



LOD = 1.2 copies/well

RCL Assay Validation Summary

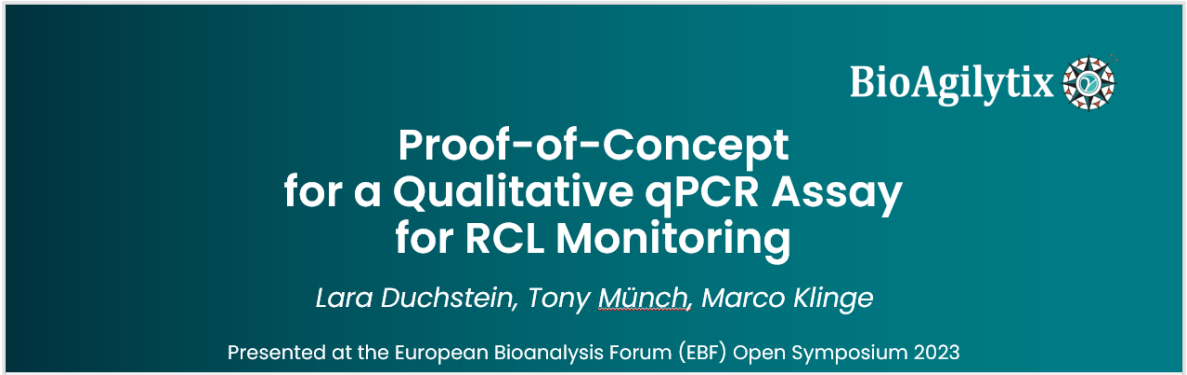
VALIDATION PARAMETER	TARGET ACCEPTANCE CRITERIA (for VSV-G)	RESULT
Linearity	$R^2 \geq 0.980$ Efficiency between 90-110%	Met acceptance criteria
Accuracy	The overall within-run and between-run accuracy for each QC level must be equal to or within ±25% (±45% at the LQC and LLOQ) of the nominal concentration.	Met acceptance criteria for between-run accuracy
Precision	Within-run and between-run precision (CV) for each QC level must be less than or equal to 25% (≤ 45% at the LQC and LLOQ).	Met acceptance criteria for between-run precision
Selectivity	A minimum of 80% of individual PBMC samples at each spike level, must have a mean replicate BCC equal to or within ±25% (±45% at the LQC) of the spiked nominal concentration with CV less than or equal to 25% (≤45%, at the LQC). A minimum of 80% of blank (unspiked) matrices must result in VSV-G signals below the LOD.	Met acceptance criteria
Specificity	VSV-G reagents must detect the VSV-G template and must show undetectable signal with ALB template.	Met acceptance criteria
Sensitivity	LOB = mean Ct value - 1.645*SD LOD = lowest spike concentration at which 95% of positive samples give a positive result (Ct < LOB) LLOQ = lowest concentration with acceptable A&P	LOB Ct = 45 LOD = 1.2 copies/well LLOQ = 10 copies/well
Range of Quantitation	ULOQ and LLOQ standards and QCs that have acceptable precision and linearity criteria (standards) or accuracy (QCs)	ULOQ = 1,000,000 copies/well LLOQ = 10 copies/well

RCL Assay Validation Summary

VALIDATION PARAMETER	TARGET ACCEPTANCE CRITERIA (for ALB)	RESULT
Linearity	$R^2 \geq 0.980$ Efficiency between 90-110%	Met acceptance criteria
Accuracy (Range of expected concentration)	Range of expected concentrations (copies/well) for 100 ng gDNA input Upper Limit / Lower Limit = mean ALB concentration ± 2.326*SD	Range = 23,941 – 35,912 copies/well
Precision (dilutional linearity)	For the dilution to be considered acceptable, the % RE of the BCC must be within ± 25% of the 20 ng/µl sample after adjusting for the dilution factor.	Met acceptance criteria
Selectivity	A minimum of 80% of blank (unspiked) matrices must result in ALB concentrations within the range established in accuracy experiment.	Met acceptance criteria
Specificity	ALB reagents must detect the ALB template and must show undetectable signal with VSV-G template.	Met acceptance criteria
Sensitivity	LOB = mean Ct value - 1.645*SD	LOB Ct = 45
Range of Quantitation	ULOQ and LLOQ standards that have acceptable precision and linearity criteria	ULOQ = 1,000,000 copies/well LLOQ = 10 copies/well

Conclusions & Outlook

- Context of Use needs to be considered for qPCR assay development and validation
- Assay range increased using qualitative approach based on the LOD
- RCL testing does not require quantitative results → "detection" sufficient
- **Qualitative RCL assay as an alternative approach**
  - For further discussion, come speak to me at my poster:



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Back-up slides

Validation Results

Dilutional Linearity – target and reference gene

- **PBMC DNA pool (40 ng/µl) diluted unspiked and at LQC spike level**
- **Acceptance Criteria**
  - % RE of the BCC must be within ± 25% of the 20 ng/µl sample after adjusting for the dilution factor

** Only 2 out of 3 wells positive*

VSV-G COPIES/WELL										
Dilution Factor	**VSV-G**					**ALB**				
	copies/well (nominal)	**copies/well (measured)**	**%CV**	**copies/well corrected for dilution factor**	**%RE**	**copies/well (nominal)**	**copies/well (measured)**	**%CV**	**copies/well corrected for dilution factor**	**%RE**
0.5	60	72.5	6.0	36.2	-19.4	60,606	71,302	0.9	35,651	5.3
1	30	44.9	23.7	44.9	0.0	30,303	33,872	3.1	33,872	0.0
2	15	17.2	28.2	34.4	-23.4	15,152	16,988	2.0	33,976	0.3
4	7.5	11.3	70.3	45.1	0.4	7,576	8,586	3.5	34,343	1.4
8	3.75	4.42	69.5	35.4	-21.2	3,788	3,989	3.2	31,908	-5.8
16	1.875	2.70 *	78.5	43.2	-3.9	1,894	1,964	3.9	31,420	-7.2

QCs

Set-up / Acceptance Criteria / Performance

- **HQC/MQC/LQC** = VSV-G and ALB spiked in PBMC pool DNA (100 ng/rxn)
- **NQC** = unspiked PBMC pool DNA freshly prepared for each run
- **NTC** = unspiked surrogate matrix
- 2 sets of QCs per plate

QC Level	Nominal Concentration of VSV-G (copies/well)	Target Plate Acceptance Criteria for VSV-G	Target Plate Acceptance Criteria for ALB	Validation Result
HQC	50,000	%RE within ± 25% Triplicate well CV for quantities ≤ 25%	Triplicate well CV for quantities ≤ 25%	Passed in 98%
MQC	2,500			Passed in 92% for VSV-G Passed in 98% for ALB
LQC	30	%RE within ± 45% Triplicate well CV for quantities ≤ 45%		Passed in 91% for VSV-G Passed in 98% for ALB
NQC	0	Negative for ≥ 2 out of 3 technical replicates		Passed in 100% for VSV-G Passed in 96% for ALB
NTC	0		Negative for ≥ 2 out of 3 technical replicates	Passed

Validation Results

ALB concentration in unspiked matrix samples

- NQC (100 ng/well = 30,303 copies/well)
- Method for Limit Calculation = Mean ± 2.326*SD
- Target Acceptance Criteria
  - %CV ≤ 25%

INTER-ASSAY MEAN (COPIES/WELL)	
	NQC (30,303)
copies/well	29,927
%CV	8.6%
Lower Limit	23,941
Upper Limit	35,912