EUFETS GmbH

Validation of a PCR for the quantification of residual plasmid DNA in samples from a biodistribution study for a therapeutic RNA vaccine

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Background

Quantitative determination of plasmid DNA (kanamycin resistance gene) from mouse organs by real-time qPCR

→ Plasmid serves as template for production of RNA vaccine
→ Plasmid remains as impurities in product
→ Residual plasmid needs to be quantified as part of a biodistribution study

Studies performed @EUFETS:
- Method development
- Validation of method (GLP)
- GLP study for sample analysis

RNA production process

Plasmid DNA serves as template for RNA production

Plasmid DNA remains as impurities in drug product

Time	Process Step
Day 1	Step 1: Plasmid Linearization Linearization of DNA template
Day 1	Step 2: DNA Purification Removal of impurities
	Hold time: 2-8°C, max. 24h
Day 2	Step 3: Transcription mRNA transcription from template incl. capping followed by DNA hydrolysation
Day 2	Step 4: mRNA Purification 1/2 Removal of impurities
	Hold time: 2-8°C, max. 24h
Day 3	Step 4 (cont.): mRNA Purification 2/2 Removal of impurities
Day 3	Step 5: 0.2 µm Filtration and Filling Reduction of bioburden

RNA products contain plasmid DNA

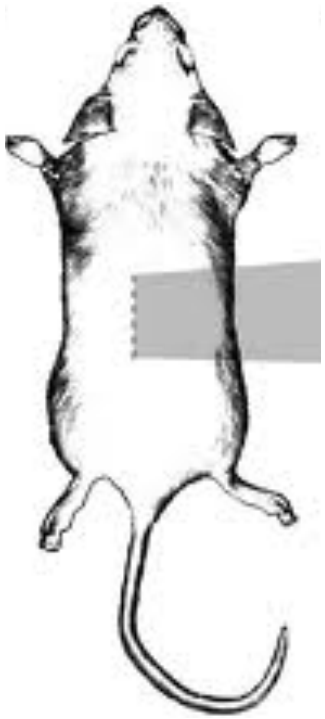
RNA #1 = 2 mg/mL RNA; 0.48 ng residual plasmid 1 DNA/mg RNA
RNA #2 = 2 mg/mL RNA; 11.8 ng residual plasmid 2 DNA/mg RNA

Both plasmids contain **kanamycin resistance gene** (**KAN**)

→Target for PCR

Plasmid#1 serves as source for standard for PCR
Concentration: 2.0 mg/mL Plasmid DNA
Calculated plasmid concentration: 3.61345 x 10e14 copies/mL

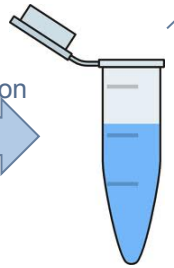
Method: sample preparation



- Blood
- Lymph node (inguinal, left/right)
- Ovary (left/right)
- Testis (left/right)

Organs/blood samples:
- *formal identification*
- *weighting of organs*

enzymatic digestion

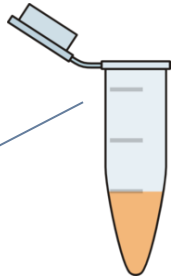


DNA isolation:
- *enzymatic digestion*
- *isolation of genomic DNA*

DNA isolation

- *photometric quantification of DNA*
- *storage of DNA (eluate) 200 µL/sample*

eluate / analyte

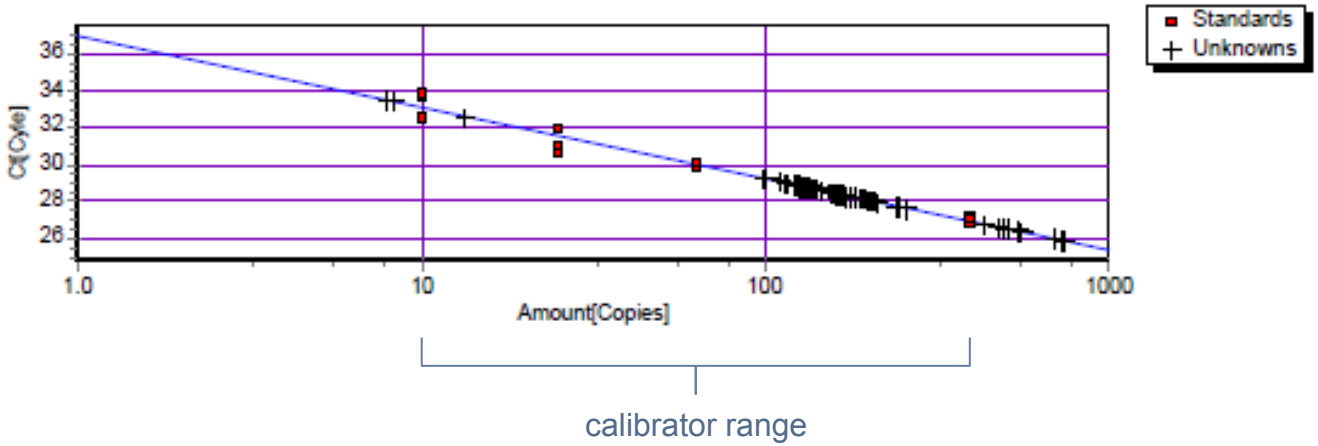


PCR:
- *analysis of 5 µL eluate as template (triplicates)*
- *result: plasmid copies/reaction*

Method: real time PCR

Quantification calibrators

- *serial dilutions prepared as stock solutions*
- *10 to 390 plasmid copies/reaction*
- *matrix: genomic mouse DNA*
- *COD must be ≥ 0.95*



Controls:

- *NTC*
- *melting curve (SYBR Green)*
- *positive control*
- *DNA isolation control*
- *EPO PCR*

Assay: contamination issue

NTC as indicator for
a) carry over, cross contamination etc. b) contamination of materials

→ omnipresence of template in lab
→ plasmid DNA stability
→ KAN / NEO homology
→ plasmid stocks
→ amplification of template
→ bacterial DNA contamination of materials (enzyme)

Assay: contamination issue

Lab organisation:

- *strict separation of DNA isolation, organ preparation, PCR preparation, amplification and removal of waste*
- *change of lab coat and gloves*
- *cleaning of surfaces, UV-lamp*

Materials:

- *selection of alternative material (polymerase)*
- *NTC: false positive indicate contamination*

Vendor	*NTC: false positive of all reactions*
QuantiTect SYBR Green Kit *(Qiagen)*	7/24
Dynamo HS SYBR Green qPCR Kit *(Finnzymes)*	0/24
Maxima SYBR Green qPCR Master Mix *(Fermentas)*	2/24
PowerSYBR Green qPCR Master Mix *(Applied Biosystems)*	5/24

Validation: Overview

Risk Analysis

SOPs:

- *DNA Isolation (organ/blood)*
- *PCR (KAN/EPO)*

Table 17 List of relevant SOP / PAN used for this validation.

Type	Title	Number	Revision
PAN	DNA-Isolierung: Solide Organe	PAN-0074-K-02	2014
PAN	DNA-Isolierung aus Blut	PAN-0075-K-02	2014
PAN	pPCR SYBR Green (KAN)	PAN-0076-K-02	2014
PAN	pPCR SYBR Green (EPO)	PAN-0087-K-02	2014
PAN	Konzentrationsbestimmung DNA	PAN-0085-K-02	2014
SOP	Abweichungsmanagment	EUF-265-D-02	2012
SOP	Rohdaten	GLP-206-S-06	2012
SOP	Mastercycler ep realplex	EUF-268-G-01	2013
SOP	UV/VIS Spektralphotometer	EUF-278-G-01	2013
SOP	Automatische Waagen	EUF-240-G-02	2013
SOP	Zentrifugen	EUF-241-G-03	2013
SOP	Umgang mit Pipetten	EUF-212-G-02	2012
SOP	Sicherheitswerkbänke	EUF-236-G-04	2013
SOP	Kühl- und Gefriergeräte	EUF-242-G-03	2013

Main equipment:

- *Qualification: Mastercycler ep realplex*

Table 15 List of equipment

Equipment	Manufacturer	Equipment number	Status of qualification	Status of calibration/ maintenance
Centrifuge 5417 C	Eppendorf	103-ZFG-02	qualified	maintenance service
Centrifuge UEC Micro 14-B	Uni Equip	113-ZFG-03	-	-
Pipette Reference	Eppendorf	-	-	calibrated
Pipette Research	Eppendorf	-	-	calibrated
Pipette Multipette Plus	Eppendorf	-	-	calibrated
Freeezer	Liebherr	113-TKS-01	-	-
Freezer	Liebherr	103-TKS-02	-	-
Heating device HTM 130-LF	HLC	103-DIV-06	-	calibrated
Mastercycler Realplex 4S	Eppendorf	105-ANL-26	qualified	maintenance service
Mastercycler Realplex 4S	Eppendorf	105-ANL-31	qualified	maintenance service
Refrigerator Liebherr FKU 1800	Liebherr	113-KSK-02	-	-
Refrigerator Liebherr Premium	Liebherr	103-KSK-02	-	-
UV VIS Spectral photometer Specord 50PC	Analytik Jena	105-ANL-28	qualified	maintenance service
UV-Lamp	Uni Equip	103-DIV-10	-	-
Vortex Genie 2 G 560 E	Scientific Industries	103-DIV-07	-	-
Vortex Genie 2 X 3	Scientific Industries	103-DIV-08	-	-
Vortex MS2	IKA	113-DIV-07	-	-
Vortex Reaxtop	Heidolph	103-DIV-09	-	-

Parameter:

- *LLOD*
- *Linearity*
- *Accuracy*
- *Precision*
- *Range*
- *Recovery*
- *Repeatability*
- *Robustness*
- *Specificity*

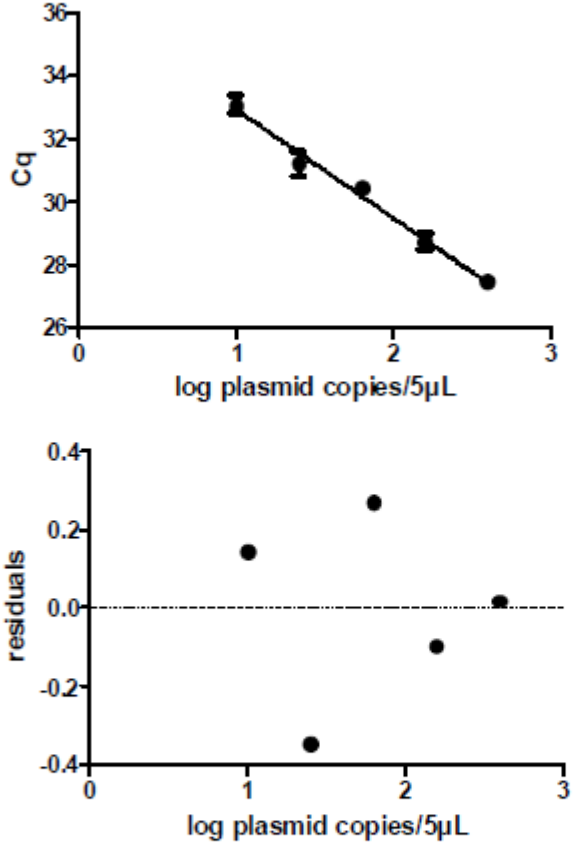
Validation: Linearity

Acceptance criteria:

- *slope of the calibration function has to differ from 0 significantly*
- *residual of Cq values of the calibration curve (residual = observed – predicted value) the variation has to behave randomly*
- *r2 (COD) of the linear function ≥ 0.95*

Determination:

- *data from each calibration curve*



Validation: LLOD

Definition

- *lowest value detected by the method but must not be determined quantitatively*
- *Cq < 40 considered positive*

Determination

- *LLOD test items were prepared by serial dilution. For dilution elution buffer containing 150, 50 and 10 µg/mL mouse DNA were used*
- *LLOD test items were measured in 6 assays in quadruplicates (n = 24)*

Acceptance criterion:

- *LLOD was defined as the analyte concentration where 95% of the measurements showed positive response*

Validation: LLOD

Results

- *distinctly below the lowest standard*
- *mouse DNA matrix showed influence on LLOD*

	150 µg/mL mouse DNA matrix	50 µg/mL mouse DNA matrix	10 µg/mL mouse DNA matrix
Plasmid copies/ reaction	**7.5**	**5**	**3.25**
Plasmid copies/µg DNA	**6**	**20**	**65**

Validation: Intermediate Precision

Determination:

- *test items were prepared from highest standard by dilution in elution buffer containing mouse DNA*
- *156.4, 78.2, 62.6 and 39.1 plasmid copies/reaction*
- *2 operators x 3 determinations; n = 6*
- *mean/SD/%CV*

Acceptance criterion:

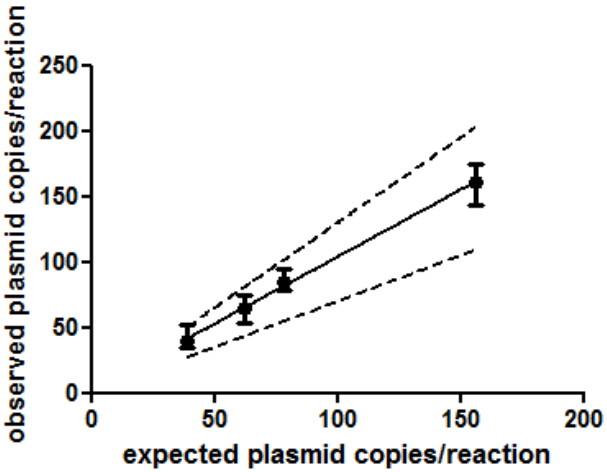
- *CV < 25%*

Validation: Accuracy

Recovery rate:
- *observed value / expected value x 100 [%]*

Determination
- *recovery rate of all data obtained during precision*

Acceptance criteria
- *recovery rate has to be in between 75% and 125%*

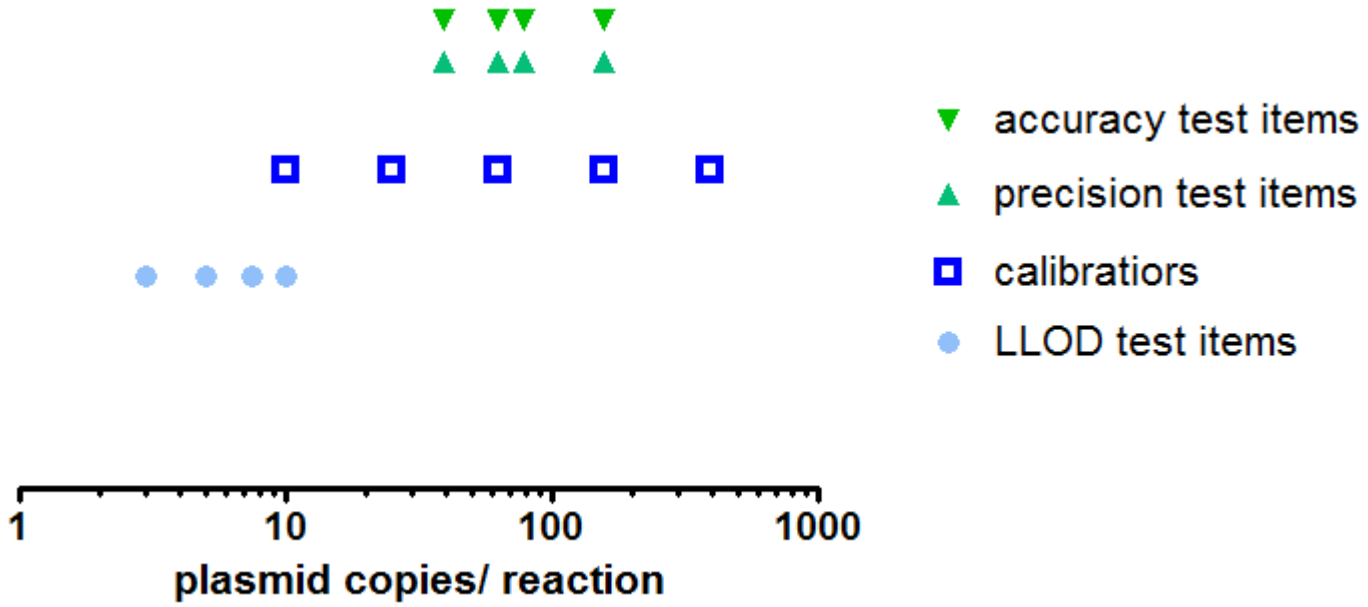


recovery rate:
- *156.4 plasmid copies/reaction* ✓
- *78.2 plasmid copies/reaction* ✓
- *62.6 plasmid copies/reaction* ✓
- *39.1 plasmid copies/reaction* *1/6* *NO*

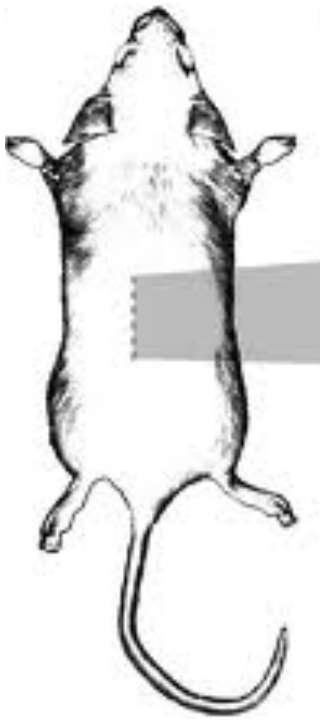
Validation: Range

Definition:

- *defined by inclusion of all concentration levels that have met the described acceptance criteria for precision, accuracy and linearity*



Validation: Repeatability



- Blood #1
- Blood #2
- Blood #3
- Blood #4

3 x 75 µL/blood sample

plasmid spike

Enzymatic digestion

DNA isolation

eluate / analyte

Determination:

- *repeatability test items were prepared using spiked blood samples*

Validation: Repeatability

Determination:

- *repeatability test items were prepared using spiked blood samples*
- *separate DNA isolation and PCR for each of 3 blood samples per concentration*
- *mean/SD/% CV of three repeatability test items prepared in parallel*

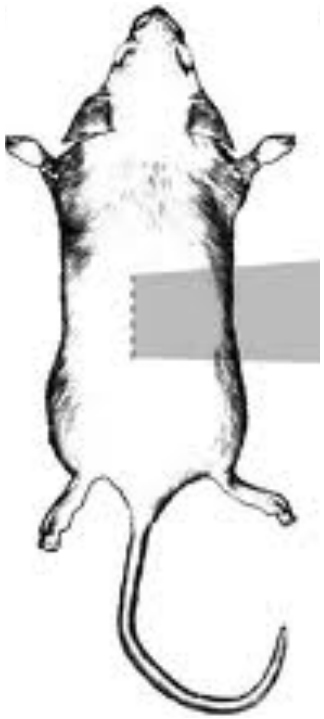
Acceptance criterion:

- *CV must be < 25%*

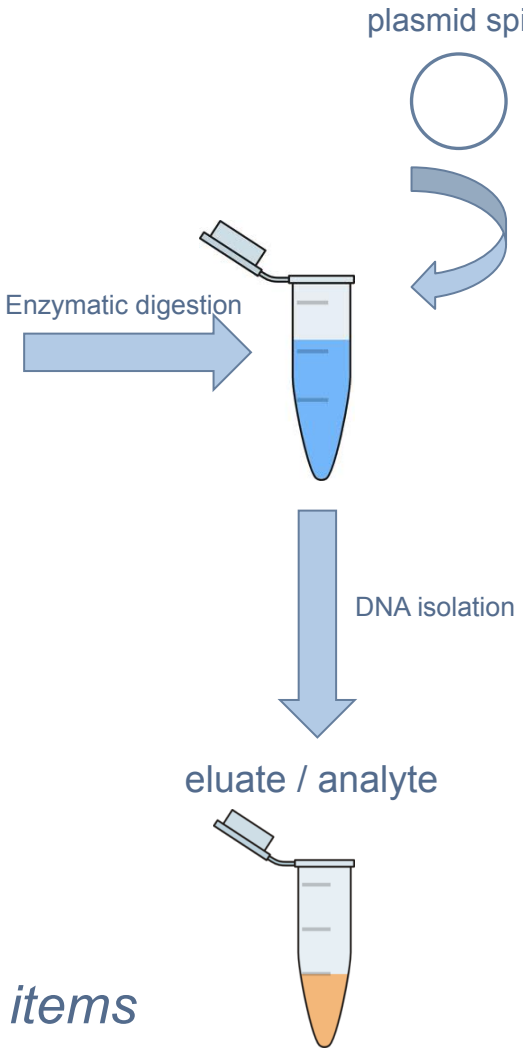
48 plasmid copies/reaction < 25%
23 plasmid copies/reaction > 25%
11 plasmid copies/reaction > 25%

0 plasmid copies/reaction < LLOD

Validation: Recovery



- Blood
- Lymph node (inguinal, left/right)
- Ovary (left/right)
- Testis (left/right)



Determination:

- *spiked organ samples as recovery test items*

Validation: Recovery

Determination:

- *spiked organ samples as recovery test items*
- *reference specimen were prepared in elution buffer containing 150 µg/mL mouse DNA representing 100 % of the analyte at the respective concentration in matrix (mouse DNA)*

Acceptance criteria:

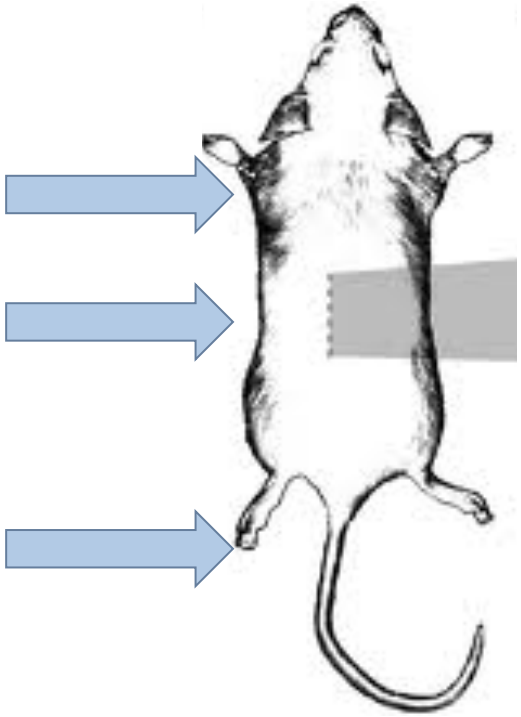
- *none*
- *recovery is reported only*

Biodistribution study: Overview

Intranodal (i.n.)
Right inguinal lymph node: RNA #1
Left inguinal lymph node: RNA #2

Intranodal with perfusion
(data not shown)

Intraveneous (i.v.)
RNA #1 and RNA #2

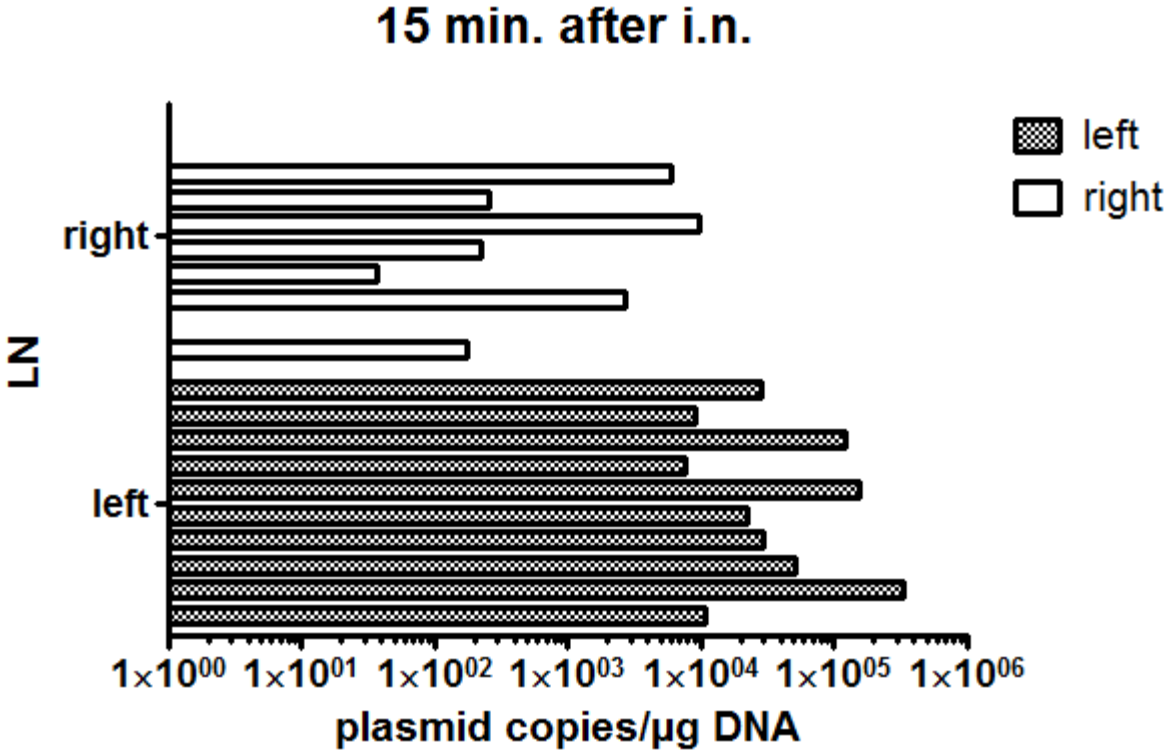


Samples = 400

- Blood
- Lymph node (inguinal, left/right)
- Ovary (left/right)
- Testis (left/right)

Organ sampling after administration:
→ *15 min.*
→ *24 h*
→ *7 days*

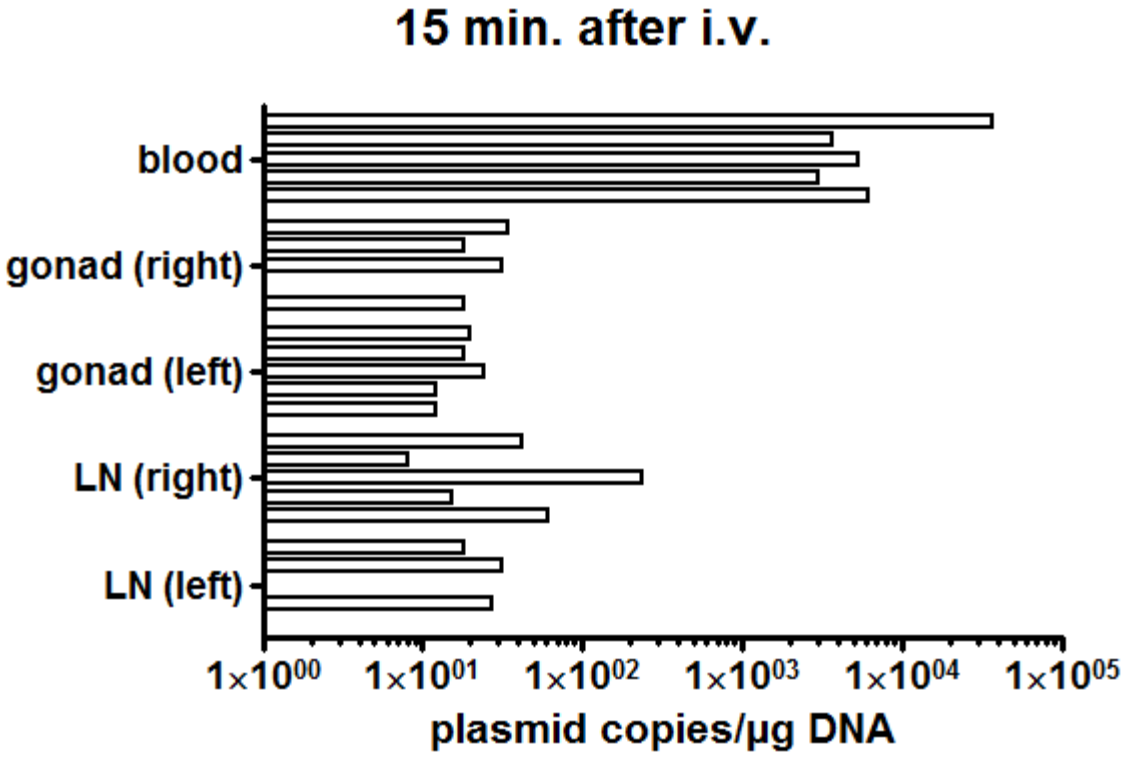
Biodistribution study: Results



Right inguinal lymph node: *RNA #1* 0.48 ng residual plasmid DNA/mg RNA
Left inguinal lymph node: *RNA #2* 11.8 ng residual plasmid DNA/mg RNA

→24 h: 1 positive LN (of 10); no positive gonad or blood samples
→7 days: no positive samples

Biodistribution study: Results



→24 h: no positive samples
→7 days: no positive samples

Thank you for your attention !