VIRAL RNA ISOLATION

For the titration of lentiviral particles the use of **NucleoSpin RNA Virus kit** is recommended (Macherey-nagel). Indeed NucleoSpin RNA Virus is designed for the isolation of viral nucleic acids from serum, plasma, or any cell-free biological fluids.

For the complete protocol visit the website:

http://www.mn-net.com/Portals/8/attachments/Redakteure_Bio/Protocols/Viral%20nucleic%20acids/UM_ViralRNA.pdf

Briefly:

Before starting the preparation:

- Check if Lysis Buffer RAV1 and Wash Buffer RAV3 were prepared according to section 3 of the protocol.
- Preheat an aliquot of Elution Buffer RE/ RNase-free H2O to 70 °C.
- Add 600 µL Buffer RAV1 containing Carrier RNA to 150 µL of the sample. Pipette mixture up and down and vortex well. Incubate for 5 min at 70 °C.
- Add 600 µL ethanol (96–100 %) to the clear lysis solution and mix by vortexing (10–15 s).
- Place NucleoSpin® RNA Virus Columns in Collection Tubes (2 mL) and load 700 µL lysed sample. Centrifuge for 1 min at 8,000 x g.
- Add 500 µL Buffer RAW to the NucleoSpin® RNA Virus Column. Centrifuge for 1 min at 8,000 x g. Discard flowthrough (1 wash).
- Add 600 µL Buffer RAV3 to the NucleoSpin® RNA Virus Column. Centrifuge for 1 min at 8,000 x g. Discard flowthrough with Collection Tube (2 wash).
- Place the NucleoSpin® RNA Virus Column in a new Collection Tube (2 mL) and add 200 µL Buffer RAV3. Centrifuge for 2–5 min at 11,000 x g to remove ethanolic Buffer RAV3 completely (3 wash).
- Place the NucleoSpin® RNA Virus Column into a new, sterile 1.5 mL microcentrifuge tube (not provided). Add 50 µL RNase-free H2O (preheated to 70 °C) and incubate for 1–2 min. Centrifuge for 1 min at 11,000 x g.

LENTIVIRAL TITRATION IS PERFORMED BY USING THE **Lenti-X™ qRT-PCR Titration Kit** (CLONTECH):

http://www.clontech.com/US/Products/Viral_Transduction/Lentiviral_Transduction_Tools/Lentivirus_Titration/qRT-PCR

The protocol for virus titration can be found at the following link:

http://www.clontech.com/IT/Products/Viral_Transduction/Lentiviral_Transduction_Tools/Lentivirus_Titration/ibcGetAttachment.jsp?cItemId=17574&fileId=6731500&sitex=10023:22372:US

Autor: S. Di Franco **22/12/2016** **Approved: Giorgio Stassi**

Briefly:

- Harvest lentiviral supernatant from cells and centrifuge 5 min at 2000 rpm to remove cells and debris.
- Aliquot 200 µl of supernatant for immediate titration, or store at –80°C.
- Purify RNA from 150 µl of supernatant using the NucleosSpin RNA Virus Kit according to the protocols contained in the Viral RNA Isolation User Manual. Smaller volumes (5–100 µl) can also be used if necessary.
- Elute the RNA in 50–100 µl RNase-free water.
- If your lentivirus was produced from a stable, lentivirus-producing cell line and not from a transient transfection, DNase I treatment (shown below) is optional, and you may proceed directly to qRT-PCR (Section C). If your lentivirus was produced from a transiently transfected packaging cell line, residual plasmid DNA must be removed prior to qRT-PCR. Treat these types of viral RNA sample(s) with a DNase I reaction as shown in Table II:

Table II: DNase I Reaction

Reagent[1]	Volume (µl)
RNA Sample	12.5
DNase I Buffer (10x)	2.5
DNase I (5 units/µl)	4.0
RNase-Free Water	6.0
Total	**25.0**

1 An RNase inhibitor may be included in the DNase I reaction but is generally unnecessary.

- Combine reagents, mix, and incubate at 37°C x 30 min, followed by 70°C x 5 min. A thermocycler should be used for this reaction. Store the tubes on ice until ready to perform qRT-PCR.
- In your reaction assembly work area, and on ice, assemble a sufficient volume of Master Reaction Mix (MRM) using the reagents shown in Table III. Add the RT Enzyme Mix last. To ensure that you will have enough MRM, prepare approximately 10% more than the minimum required for your experiment. Each control, no-template control (NTC), and sample reaction should be performed in duplicate:

Table III: Master Reaction Mix for qRT-PCR

Reagent	Volume/well (µl)	Total wells	Total volume	Total + 10%
RNase-Free Water	8.5 (8.0)			
Quant-X Buffer (2X)	12.5			
Lenti-X Forward Primer (10 µM)	0.5			
Lenti-X Reverse Primer (10 µM)	0.5			
ROX Reference Dye LSR or LMP (50X)[1]	(0.5)			
Quant-X Enzyme	0.5			
RT Enzyme Mix	0.5			
Total	**23.0**			

1 The kit is supplied with two different ROX formulations that allow you to normalize fluorescence signals on instruments that are equipped with this option. ROX Reference Dye LSR is for instruments whose excitation source is a 488 nm laser, while ROX Reference Dye LMP is for instruments whose excitation source is either a lamp or an LED. Be certain to use the formulation that is appropriate for your real-time instrument!

Example of a basic experiment with minimum MRM volumes: • Controls: 5 serial dilutions in duplicate; 10 wells x 23 µl = 230 µl MRM. • NTCs: 3 each in duplicate; 6 wells x 23 µl = 138 µl MRM. • Samples: 4 dilutions in duplicate; 8 wells x 23 µl = 184 µl MRM/sample

- In your sample dilution work area, and using PCR grade 8-well strips, construct a standard curve of Lenti-X RNA Control Template dilutions, and make serial dilutions of your purified viral RNA sample(s) as described below and shown in Table IV:

Table IV: Control and Sample Dilutions for qRT-PCR[1]

	Strip 1: Controls			Strip 2+: Samples		
Well	EASY	Additive[2]	Amount[3]	EASY	Additive[2]	Amount
1	18	2	5×10^7	–	20	Sample 1: 1x
2	27	3	5×10^6	27	3	0.1x
3	27	3	5×10^5	27	3	0.01x
4	27	3	5×10^4	27	3	0.001x
5	27	3	5×10^3	–	20	Sample 2: 1x
6	10	–	NTC	27	3	0.1x
7	10	–	NTC	27	3	0.01x
8	10	–	NTC	27	3	0.001x

1 2 µl of each control and sample dilution will be used in each qRT-PCR reaction
2 See protocol for additive source.
3 copies/µl

- Add EASY Dilution Buffer to the appropriate wells of the strips, as shown in Table IV. NTCs in wells 6–8 of Strip 1 contain only EASY Dilution Buffer.
- In wells 1–5 of Strip 1, prepare 10-fold serial dilutions of the Lenti-X RNA Control Template as follows:

a. In well 1, dilute 2 µl of the Lenti-X RNA Control Template stock into 18 µl of buffer for a 1:10 dilution (10X stock = 5×10^8 copies/µl).

b. In wells 2–5, perform 10-fold serial dilutions of the diluted control template in well 1 by transferring 3 µl of well 1 into the 27 µl of buffer in well 2. Repeat similar dilutions for wells 3–5.

- Serially dilute your viral RNA samples as shown in Table IV. Each 8-well strip can be used for 2 samples at 4 different concentrations each.

a. The first well in each series (wells 1 & 5) should contain 20 µl of undiluted sample (1x).

b. Subsequent 10-fold sample dilutions (wells 2–4 & wells 6–8) can be made by serially transferring 3 µl of the preceding dilution into 27 µl of buffer in the next well.

- Mix well by tapping gently, and centrifuge the strips at 2000 rpm (4°C) for 1 min to remove any bubbles.
- In your qPCR reaction assembly area, place a 96-well PCR plate on ice (or a blueblock; 4°C), and dispense 23 µl/well of MRM into the appropriate wells (in duplicate) using a repeating pipet.

- Using a multichannel pipet, transfer 2 µl/well of the control dilutions, NTCs, and sample dilutions (in duplicate) from the 8-well PCR strips to the PCR plate containing MRM.
- Centrifuge the plate at 2000 rpm (4°C) for 2 min to remove any bubbles.
- 10. Program your real-time qPCR instrument for the following qRT-PCR reaction cycles. Include a final dissociation curve cycle. Install the plate in the instrument and start the program.

- **RT Reaction**
  - 42°C 5 min
  - 95°C 10 sec
- **qPCR x 40 Cycles**
  - 95°C 5 sec
  - 60°C 30 sec
- **Dissociation Curve**
  - 95°C 15 sec
  - 60°C 30 sec
  - All (60°C–95°C)

Data Analysis

1. Determine average Ct values from the control dilution duplicates and plot vs. copy number (log scale) to generate a standard curve (Figure 2).

2. Determine average Ct values for each duplicate sample dilution and read the corresponding copy number value from the standard curve. Use all Ct values that are below that of the NTC.

3. For each dilution, back-calculate a starting copy number value for the original sample using the example given below. Generate a mean value to determine the RNA genome content of the sample.

Calculating RNA copy numbers and infectivity coefficients, an example:

1. **Copy numbers:** 150 µl of a sample was purified and eluted in 50 µl. The undiluted sample corresponded to a raw copy number of 1 x 10^7 copies on the qRT-PCR Standard Curve.
   - Copies/ml = $\frac{(1 \times 10^7 \text{ copies})(1000 \text{ µl/ml})(2\text{x DNase})(50 \text{ µl elution*})}{(150 \text{ µl sample*})(2 \text{ µl added to well})}$
   - Copies/ml = 3.33 x 10^9

   *These values are user defined.

   Note: NTC values average ~35 C_t in our experiments
2. **Infectivity coefficients:** If you have also determined viral infectivity (i.e. via FACS®), calculate an RT:FACS ratio (copies/IFU), or infectivity coefficient, for your virus by dividing the qRT-PCR copies/ml by the IFU/ml value from your FACS titration. This coefficient can then be used to calculate the IFU/ml for subsequent qRT-PCR titration results.
   - Using the copy number from the above example (3.33 x 10^9) and an RT:FACS ratio from Table I, p. 3 (93), IFU/ml = (3.33 x 10^9 copies/ml)/(93 copies/IFU)
   - IFU/ml = 3.6 x 10^7

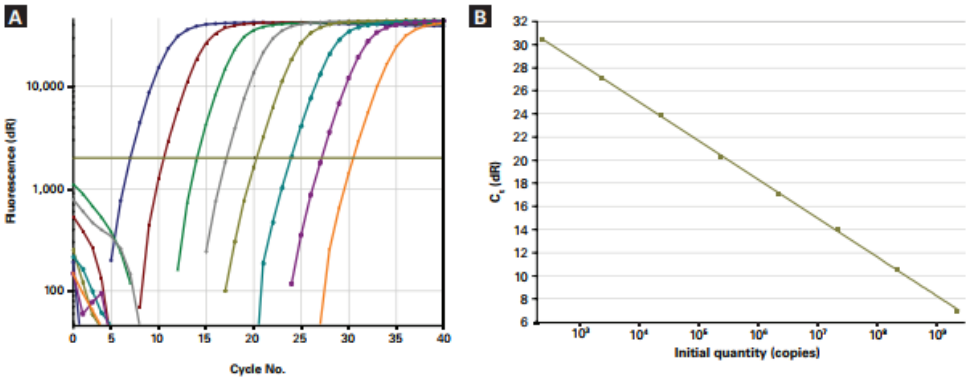

Figure 2. Using the Lenti-X RNA Control Template to generate a standard curve. Panel A. Amplification plots of a qRT-PCR reactions using serial dilutions of the Lenti-X RNA Control Template (10^9–10^2 copies) and the Lenti-X qRT-PCR Titration Kit. The assay shows a dynamic range of at least six orders of magnitude with low No Template-Control background (not shown). **Panel B.** A standard curve created from the plots shown in Panel A demonstrates a strong linear correlation between the C_t values and the RNA copy numbers (log scale), with R^2 = 1.00 and a PCR efficiency of 99.3%.