CYGNUS TECHNOLOGIES
part of Maravai LifeSciences

MockV® RVLP Kit

Product Guide

Catalog # M230, M231-1, M100, M105

PRINCIPLE OF THE KIT

This kit provides a stock solution of CHO-cell derived Retrovirus Like Particles (RVLPs) and quantification components to analyze their removal from biopharmaceutical feedstreams through downstream process steps. The analytical assay consists of an RNA extraction step and a qPCR step.

REAGENTS & MATERIALS PROVIDED

Component	Catalog #	Quantity/Volume	Storage
Deep Well Extraction Plate with Sealing Mat	M102-1	1	Room Temperature
RVLP Stock Solution	M231-1	1 x 2.0 mL	4°C
RVLP Processing Buffer	M007-15	1 x 15 mL	4°C
Endonuclease	M232-1	1 x 200 µL	-20°C
Endonuclease Buffer	M233-1	1 x 500 µL	4°C
Proteinase K	M103-1	1 x 150 µL	4°C
RNA Extraction Buffer	M008-30	1 x 30 mL	4°C
RNA Precipitation Buffer	M009-55	1 x 55 mL	4°C
RNA Wash Buffer	M010-170	1 x 170 mL	4°C
RNA Reconstitution Buffer	M011-15	1 x 15 mL	4°C
RVLP Master Mix	M234-1	1 x 550 µL	-20°C
RVLP Forward Primer	M235-1	1 x 200 µL	-20°C
RVLP Reverse Primer	M236-1	1 x 200 µL	-20°C
RVLP Probe	M237-1	1 x 75 µL	-20°C
PCR Grade Water	M238-1	1 x 200 µL	-20°C
RVLP sRNA (Lyophilized)	M239-1	1 vial	-80°C

MATERIALS & EQUIPMENT REQUIRED BUT NOT PROVIDED

- Nuclease Free Water
- Several boxes of filtered micropipette tips of all sizes (2 - 1,000 µL)
- Dilution Reservoir (VWR Cat# NC9034365, or equivalent)
- Heat block with microplate adaptor
- Water bath
- Single and Multi-channel pipettes of all sizes (2 – 1,000 µL)
- Plate shaker
- Centrifuge with microplate adaptor
- Analytical balance
- Deep Well Extraction Plate for balancing the Kit's Deep Well Extraction Plate (Cygnus Cat# D102)
- qPCR instrument
- PCR hood (recommended)
- Sample Diluent Buffer (Cygnus Cat# I028)

PRELIMINARY STUDIES

Prior to performing sample analysis, **consult with Cygnus Technologies and your Analytical department** to discuss the preliminary studies or assay qualification that should be undertaken. Typically, a sample hold and spike/recovery study is performed to determine proper sample storage conditions and minimal required dilutions of each sample type.

Freeze/Thaw and Spike/Recovery Study

Certain impurities (ex. proteases) or strongly acidic/basic buffer conditions may affect the long-term stability of RVLP in samples collected during experimentation from the time they are collected to the time they are analyzed. It is recommended that samples are **frozen at -80°C in single use aliquots.** As an added precaution, a Freeze/Thaw experiment should be performed. In addition, high concentrations of therapeutic product, impurities or buffer components present in samples may inhibit the extraction of RNA from RVLP prior to RT-qPCR analysis. When such product or sample matrix interference can be demonstrated, it may be necessary to dilute samples to a Minimal Required Dilution (MRD) to achieve an acceptable recovery. Spike/Recovery experiments should be performed on each unique sample matrix ahead of sample analysis. These studies can be performed in parallel as described below.

1. Spike 10 µL of RVLP Stock Solution into 990 µL of each sample and 5 µL of RVLP Stock Solution into 495 µL of Sample Diluent Buffer (as a spiked control) achieving 1% v/v additions of each.
2. Freeze the spiked samples and control prepared during Step 1 at -80°C.
3. After a complete freeze, remove samples and controls from -80°C and thaw. To each spiked sample, prepare 500 µL of a 1 to 2 dilution and 1 to 10 dilution, using **nuclease free water** as diluent. Use Table 1 as a reference.

Table 1: Post - Thaw Sample Preparation

Sample/Control Type	Dilution	Volume of Sample/Buffer	Volume of nuclease free water
Spiked Sample	None (“neat”)	500 µL	0 µL
	2	250 µL	250 µL
	10	50 µL	450 µL
Spiked Sample Diluent Buffer Control	None (“neat”)	500 µL	0 µL

4. Prepare a fresh set of samples and controls described in Table 2.

Table 2: Fresh Sample Preparations

Sample/Control Type	Dilution	Volume of Sample/Buffer	Volume of nuclease free water	RVLP Stock Solution
Spiked Sample	None ("neat")	495 µL	0 µL	5 µL
	2	247.5 µL	247.5µL	5 µL
	10	49.5 µL	445.5 µL	5 µL
Spiked Sample Diluent Buffer control	None ("neat")	495 µL	0 µL	5 µL
Unspiked nuclease free water control	None ("neat")	NA	500 µL	0 µL
Unspiked Sample Diluent Buffer control	None ("neat")	500 µL	0 µL	0 µL

5. Test all samples/controls prepared above according to the analytical assay as described in this Product Guide. Calculate RVLP concentration values (See "Calculation of Results" section below). Ensure that any Dilution factor has been accounted for during each RVLP Concentration determination. Determine the % recovery of freeze/thawed and freshly prepared samples relative to the theoretical spiked value (1 x 10^8 RVLP/mL) and to the appropriate Sample Diluent Buffer control (frozen vs. frozen, fresh vs. fresh). Determine each sample's MRD. Acceptable recovery can be defined as 50-250%, but it is highly recommended to discuss your results and criteria with your Analytical Department and/or Cygnus Technologies.

SAMPLE STORAGE

After obtaining samples from RVLP spiking experimentation, aliquot and store them at -80°C prior to analysis. Make several small volume aliquots to ensure the ability of performing only one single freeze/thaw. If high salt is present in sample (> 200 mM), dilute to 200 mM with water and note the dilution factor. If sample exhibits low or high pH (< 5.0 or > 9.0), adjust to near neutral pH with buffer components (avoid highly concentrated HCl or NaOH). Samples can be 0.22 µm filtered prior to storage but it is preferable to use a 0.45 µm filter and add Sodium Azide to final concentration of 0.025%.

RVLP SPIKING STUDIES

1. Prepare pre-spiked load material accordingly (ex. warm to room temperature, pre-filter, etc.) and determine volume.
2. Take 5 x 500 µL samples of this pre-spiked material and freeze at -80°C.
3. Determine desired RVLP spike by:

    a) Targeting a total RVLP particle challenge (ex. 10 $\log_{10}$ Total RVLP Particles), or;
    b) Targeting a final RVLP particle concentration (ex. 1.0 x 10^8 RVLP/mL of load).

4. Determine volume of Spiking RVLP to add into load. If spiking according to "A" above, use the following formula:

    $$\text{Volume (mL)} = \frac{Target\ Total\ log10\ RVLP\ Challenge}{\text{Titer of } RVLP \text{ Stock Solution } (\frac{\text{RVLP}}{mL})}$$

    If spiking according to "B" above, use the following formula:

    $$\text{Volume (mL)} = \frac{(Target\ Final\ RVLP\ Concentration)\ x\ (Target\ Final\ Load\ Volume)}{\text{TIter of } RVLP \text{ Stock Solution } (\frac{\text{RVLP}}{mL})}$$

5. Pipette the determined volume of RVLP Stock Solution into load. Mix gently. Remove 5 x 500 µL of sample as a spiked load sample and freeze at -80°C. If a Load Hold Control sample is desired, remove an additional 5 x 500 µL and maintain the material at processing temperature.

6. Process remaining amount of spiked load through desired separation technique. Collect fraction and pool as necessary and prepare 5 x 500 µL aliquots of each for analysis.

7. Upon completion of separation technique, store all run samples (including Load Hold Control, if taken) according to the Sample Storage instructions above.

PREPARATION OF RVLP sRNA

RVLP sRNA comes supplied as lyophilized product. Prior to use in the analytical assay, remove RVLP sRNA from -80°C and place on ice. Centrifuge the tube at 10,000 rpm for 1 minute and then add RNA Reconstitution Buffer[1] by pipetting down the side of the tube. Place on ice for 5 minutes, briefly vortex at low setting and pulse centrifuge to bring liquid to bottom of tube. Place on ice. 5.0 µL aliquots can then be made and stored at -80°C for single use.

[1] Refer to the kits COA for proper volume of RNA Reconstitution Buffer.

ANALYTICAL ASSAY PROCEDURE

Prior to starting the analytical assay, spatially plan out the standards and samples that will be added to the deep well extraction plate – bearing in mind the later transfer to a qPCR plate (additional controls will be run on the qPCR plate). Typically, each sample and standard are run in triplicate. See Appendix 1 for an example plate layout.

Part I. Sample Preparation and Treatment

Estimated Time: 1 hour

Step 1: Remove Endonuclease Buffer from 4°C and Endonuclease from freezer. Perform a quick spin and place on ice. Prepare a mixture of the two components by adding 2 µL of Endonuclease to 5.0 µL of Endonuclease Buffer for each sample well to be used during the assay (don't take into account the standard wells).

Tip: Prepare 5-10% overage

Step 2: Place a deep well extraction plate on ice and pipette 7.0 µL of the mixture prepared in Step 1 into each sample well of the deep well extraction plate.

Tip: Add mixture to a reservoir and pipette out with a multichannel pipette.

Step 3: Add 125 µL of each sample to its respective deep well. If a dilution of the sample is necessary, ensure it has been diluted prior to addition. Use nuclease free water as diluent.

Step 4: Seal deep well extraction plate with sealing mat and place in 37°C water bath for 30 minutes.

Tip: Ensure tight seal of sealing mat onto deep well extraction plate. Plate roller or other tools may be used.

Tip: During incubation, warm RVLP Processing Buffer to room temperature and set heat block to 55-60°C. **DO NOT EXCEED 60°C**.

Step 5: Centrifuge deep well extraction plate at 3,200 x g for 2 minutes

Tip: Balance with secondary deep well extraction plate containing water. Label this extraction plate as "Balance" and keep it indefinitely for this purpose.

Part II: RNA Extraction

Estimated Time: 2 hours

Step 6: Carefully peel off sealing mat and add 125 µL of RVLP Processing Buffer to each deep well containing sample. Mix by pipetting up and down.

Step 7: Seal the plate with sealing mat (ensuring tight seal as discussed above) and shake for 5 minutes on plate shaker set to 300 rpm.

Step 8: Prepare a 1:10 dilution of Proteinase K by adding 1.25 µL of Proteinase K to 11.25 µL of RNA Reconstitution Buffer per sample well (don't take into account the standard wells).

Tip: Prepare 5-10% overage

Step 9: Add 12.5 µL of 1:10 Proteinase K to each sample well of the deep well extraction plate. Mix by pipetting up and down.

Tip: Add mixture to a reservoir and pipette out with a multichannel pipette.

Step 10: Seal the plate with sealing mat (ensuring tight seal as discussed above) and shake for 30 seconds on plate shaker set to 800 rpm.

Step 11: Incubate plate at 60°C for 30 minutes in a dry heat block with a microplate adaptor.

Tip: During incubation, thaw sRNA and perform Step 13. Warm RNA Extraction Buffer, RNA Precipitation Buffer, and RNA Wash Buffer to room temperature.

Step 12: Centrifuge deep well extraction plate at 3,200 x g for 2 minutes.

Tip: Adjust the "Balance" deep well plate by removing or adding water.

Step 13: Dilute 2 µL of reconstituted RVLP sRNA with 198 µL of nuclease free water.

Step 14: With this 1:100 diluted sRNA from Step 13 as the "starting solution", prepare a dilution series in an 8 well dilution reservoir as seen in Table 3.

Table 3: sRNA Dilution series

Dilution Point	Solution	Volume (µL)	Diluent (nuclease free water) volume (µL)	Conc. (copies/µL)
1	"Starting Solution"	2.37	997.63	5.00×10^{7}
2	Dilution Point 1	50	950	2.50×10^{6}
3	Dilution Point 2	50	950	1.25×10^{5}
4	Dilution Point 3	50	950	6.25×10^{3}
5	Dilution Point 4	50	950	3.13×10^{2}
6	Dilution Point 5	50	950	1.56×10^{1}
7	Dilution Point 6	50	950	7.81×10^{-1}
8	Diluent Only	0.00	1000	0.00

Tip: Add nuclease free water to each well prior to sRNA containing solutions. Change tip after each addition. Handle sRNA and anything that comes into contact with sRNA with care as the qPCR assay is very sensitive. Change gloves after handling.

Step 15: Carefully peel off sealing mat and add 250 µL of RVLP sRNA dilution series to each column of deep well plate designated as a standard column with multichannel pipette.

Step 16: Using multichannel pipette, add 250 µL of RNA Extraction Buffer to samples and standards. Pipette up and down several times during each addition. Change tips after each column.

Tip: Swirl the bottle containing RNA Extraction Buffer prior to use as RNA carrier may fall out of solution during storage.

Step 17: Seal the plate with sealing mat (ensuring tight seal as discussed above) and shake for 5 minutes on plate shaker set to 800 rpm. Optional: centrifuge the plate for 2 minutes *at* 3,200 x g after shaking

Tip: During the 5 minutes, warm RNA Reconstitution Buffer to 50°C and set centrifuge temperature to 4°C.

Step 18: Carefully peel off sealing mat and using multichannel pipette, add 500 µL of RNA Precipitation Buffer to samples and standards. Pipette up and down several times during each addition. Change tips after each column.

Step 19: Seal the plate with sealing mat (ensuring tight seal as discussed above) and shake for 15 minutes on plate shaker set to 800 rpm.

Step 20: Place the plate in a -20°C freezer for a minimum of 30 minutes and maximum of 2 hours.

Step 21: Centrifuge deep well extraction plate at 3,200 x g for 20 minutes at 4°C.

Tip: Adjust the "Balance" deep well plate by removing or adding water. After this step, the temperature of the centrifuge can be re-set to room temperature.

Step 22: Carefully peel off sealing mat and decant liquid over sink. Remove additional liquid by tapping the plate upside down on lint-free wipes until free of visible liquid.

Step 23: Using multichannel pipette, add 800 µL of RNA Wash Buffer to samples and standards. Seal the plate with sealing mat (ensuring tight seal as discussed above) and shake for 10 minutes on plate shaker set to 800 rpm.

Step 24: Centrifuge deep well extraction plate at 3,200 x g for 5 minutes.

Tip: Adjust the "Balance" deep well plate by removing or adding water.

Step 25: Carefully peel off sealing mat and decant liquid over sink. Remove additional liquid by tapping the plate upside down on lint-free wipes until free of visible liquid.

Step 26: Using multichannel pipette, add 800 µL of RNA Wash Buffer to samples and standards. Seal the plate with sealing mat (ensuring tight seal as discussed above) and shake for 5 minutes on plate shaker set to 800 rpm.

Step 27: Centrifuge deep well extraction plate at 3,200 x g for 5 minutes.

Tip: Adjust the "Balance" deep well plate by removing or adding water.

Step 28: Carefully peel off sealing mat and decant liquid over sink. Remove additional liquid by tapping the plate upside down on lint-free wipes until free of visible liquid.

Step 29: Using multichannel pipette, add 125 µL of heated RNA Reconstitution Buffer to samples and standards. Mix by pipetting up and down.

Tip: Attempt to visually confirm, well by well, that the RNA pellet is being resuspended while pipetting up and down. Wiggling the tip along the bottom of the well while pipetting up and down may assist.

Step 30: Seal the plate with sealing mat (ensuring tight seal as discussed above) and shake for 10 minutes on plate shaker set to 800 rpm.

Step 31: Centrifuge deep well extraction plate at 2,000 rpm for 1 minute.

Tip: Adjust the "Balance" deep well plate by removing or adding water.

Tip: Deep well plate containing samples can be frozen at -80°C until Part III (RT-qPCR). During day of analysis, remove from -80°C, thaw (37°C for 20 minutes can be utilized), shake for 5 minutes on plate shaker set to 800 rpm, centrifuge at 2,000 rpm for 1 minute.

Part III: RT-qPCR

Estimated Time: 2 hours.

Step 32: Prepare PCR Mix according to Table 4 below:

Table 4: PCR Mix

Reagent	Volume/well	Total Volume Needed[1]
RVLP Master Mix	5 µL	
RVLP Forward Primer	1.6 µL	
RVLP Reverse Primer	1.6 µL	
RVLP Probe	0.4 µL	
PCR Grade Water	1.4 µL	

1 Multiply Volume/well for each reagent by number of required wells (factoring in additional wells for pipetting allowance).

Step 33: Pipette 10 µL of resulting PCR Mix into each well to be used of a qPCR plate (see Appendix 2).

Step 34: Carefully peel off sealing mat of the deep well plate and using a multichannel pipette, add 10 µL from each deep well column to each qPCR plate column. Add 10 µL of PCR water to No Template Control (NTC) wells of qPCR plate.

Tip: Be very careful when peeling off sealing mat prior to qPCR. Change gloves, etc.

Step 35: Seal qPCR plate with optical plate sealer and centrifuge at 2,000 rpm for 2 minutes.

Step 36: Run RT-qPCR assay using the following parameters in Table 5 (40 cycles).

Table 5: RT-qPCR parameters

Stage	Temperature	Time
Hold 1	50°C	10 min
Hold 2	95°C	5 min
Cycling 1	95°C	3 seconds
Cycling 2	55°C	30 seconds
Cycling 3 (Data collection on)	72°C	30 seconds

Tip: Master Mix provided contains Low Rox reference dye. Select "NONE" reference dye if your system required High Rox. Choose FAM reporter and MGB—NFQ quencher.

The deep well plate containing your extracted RNA can be frozen at -80°C and thawed once for additional analysis. If multiple rounds of additional testing may be performed, aliquot ~ 25 µL from deep well plate into several additional 96 well plates. Seal and store at -80°c.

CALCULATION OF RESULTS

qPCR reports the number of cycles required to reach a fluorescent threshold (Ct value) which the qPCR instrument recognizes as signal. This value is inversely proportional to the amount of "amplicon" contained in the starting sample. For example, higher amounts of amplicon contained in the sample would require fewer PCR cycles to reach a fluorescent threshold (lower Ct values). Your qPCR instrument automatically calculates a threshold value of fluorescence for the entire data set and establishes a baseline level of fluorescence "noise" for each individual sample. Depending on the quality of the data you've obtained, it may be beneficial to manually adjust the threshold and/or baseline values. It may also be beneficial to look at the amplification curves and multicomponent plots on your qPCR instrument for the sample set. Odd amplification curves or fluorescent traces may not reflect true PCR amplification and any Ct value derived from such curves may be worthy of elimination from your dataset. Consult with your Analytical Department, instrument vendor and/or Cygnus if you'd like assistance. Whether using your instruments software or calculating manually on a spreadsheet, determine the average Ct values and standard deviation from your dilution series standards, samples, and qPCR controls. Determine the coefficient of variation for each data set by dividing the standard deviation by the average Ct value and express this value as a percentage. Scan these standard deviation values. If any value is > 5%, look at the amplification and multicomponent plots for the samples in question and eliminate any clear non-amplification generated values. If all curves look acceptable, consider the removal of any statistical Ct outliers from your triplicate set, then re-determine the average Ct and standard deviation. Never remove more than one outlier from an n=3 dataset. Next, look at the values achieved for your NTC qPCR control.

If the average Ct is < 37, there may have been aerosolization of amplicon during the qPCR plate set-up or a contamination of one or more PCR reagents.

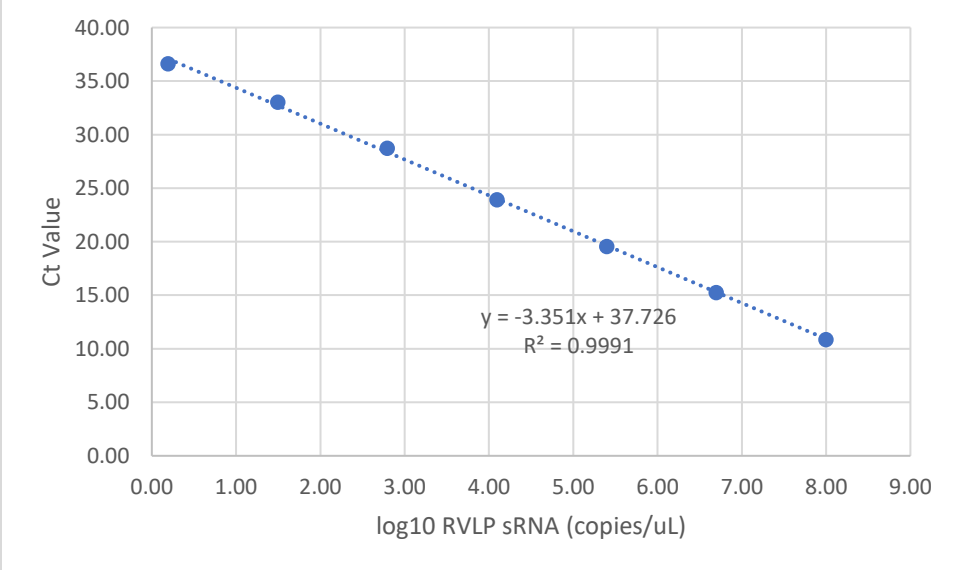
A dilution series of RVLP sRNA was run alongside the unknown experimental samples. From this series, a standard curve will be generated. First, determine if the average Ct value from Dilution point 7 is significantly different from Dilution point 8 (water only). If so, proceed. If not, remove Dilution Point 7 from further analysis. Next, convert each RVLP sRNA concentration point to a $\log_{10}$ value and plot them (X axis) against their respective average Ct values (Y axis). Do not include Dilution Point 8 in this analysis or Dilution Point 7 if it was determined to be not significantly different from Dilution Point 8. Add a line of best fit to the dataset and display the equation/r^2 value. An example is provided in Table 6 and Figure 1 below.

Table 6: sRNA Dilution series data

Dilution Point	sRNA Conc. (copies/µL)[1]	$\log_{10}$ Conc. (copies/µL)	Average Ct	Std. Dev.	CV
1	1.00×10^8	8.0	10.85	0.44	4.05%
2	5.00×10^6	6.7	15.25	0.60	3.94%
3	2.50×10^5	5.4	19.55	0.52	2.70%
4	1.25×10^4	4.1	23.90	0.54	2.27%
5	6.25×10^2	2.8	28.73	0.32	1.12%
6	3.13×10^1	1.5	33.05	0.57	1.74%
7	1.56×10^0	0.2	36.62	0.32	0.90%
8	0.00	0.0	37.83	0.89	2.35%

1. These values differ from those in Table 3. A 2X concentration factor must be applied since 250 µL of sRNA was added to the deep well plate in step 15 and reconstituted in 125 µL of RNA Reconstitution Buffer.

Figure 1: sRNA Dilution series line of best fit



Note the slope of the equation. In theory, when 100% efficiency is achieved, each qPCR cycle doubles the amount of amplicon. Therefore, for each 10^1 copy increase (unit along the X axis), the Ct value (Y axis) should shift by -3.3.

Now, the RNA concentration extracted from unknown samples can be back calculated from the equation of the RVLP sRNA dilution series line of best fit. If any average Ct value from unknown samples is lower than the average Ct value of the final RVLP sRNA Dilution Point used in generating the line of best fit, the average Ct value of that final Dilution Point should be used.

After calculating the RNA concentration from unknown samples (copies/µL), the concentration of RVLP (RVLP/mL) can be determined by:

1. Converting the $\log_{10}$ copies/µL RNA concentration to a non-logarithmic value by raising 10 to the determined $\log_{10}$ power (ex. 6.0 $\log_{10}$ = 10^6 = 1 x 10^6)
2. Multiplying by 1,000 (converting µL to mL)
3. Dividing by 2 (2 copies of RNA/RVLP)
4. Multiplying by any dilution factor use during sample preparation.

Examples of calculating the RVLP concentration of unknown samples based on the equation of Figure 1 is shown in Table 7.

Table 7: RVLP concentration Calculation

Sample Type	Avg. Ct	$\log_{10}$ Conc. (copies/µL)	RVLP Conc. (RVLP/mL)	Dilution Factor	Final RVLP Conc. (RVLP/mL)
Spiked AEX Load	19.20	5.53	1.69 x 10^8	1	1.69 x 10^8
AEX FlowThrough	37.99[1]	≤ 0.33	≤ 1.07 x 10^3	1	≤ 1.07 x 10^3
AEX Strip	25.63	3.61	2.04 x 10^6	10	2.04 x 10^7

1. Since the Ct value of 37.99 is above the Ct of the final sRNA Dilution Point used to generate the line of best fit (Dilution Point 7), the average Ct value of Dilution Point 7 (36.62) was used for further calculations and an inequality sign is used to express the results.

Log Reduction Values (LRV) can then be calculated as shown in the equation below where *C* is the Final RVLP concentration, *V* is the volume of the original fraction from which a sample was taken for analysis, and *l* or *p* denote Load or Pool, respectively (Pool is a loose term that can apply to whichever fraction it is desired to calculate an LRV for. Ex. AEX flowthrough, Protein A elution pool, etc.).

$$LRV = log_{10}\left\{\frac{(Cl\ x\ Vl)}{(Cp\ x\ Vp)}\right\}$$

Based on the example highlighted in Table 7, LRVs could be calculated as follows:

Table 8: LRV Calculation

Sample	Final RVLP Conc. (RVLP/mL)	Fraction Volume (mL)	Total RVLP	LRV
Spiked AEX Load	1.69×10^{8}	93.5	1.58×10^{10}	N/A
AEX FlowThrough	$\leq 1.07 \times 10^{3}$	80.8	$\leq 8.63 \times 10^{4}$	≥ 5.26
AEX Strip	2.04×10^{7}	12.4	2.52×10^{8}	1.80

APPENDICES

Appendix 1: Sample Deep well Extraction Plate Layout

Standard 1	Standard 1	Standard 1	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17
Standard 2	Standard 2	Standard 2	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18
Standard 3	Standard 3	Standard 3	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19
Standard 4	Standard 4	Standard 4	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20
Standard 5	Standard 5	Standard 5	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21
Standard 6	Standard 6	Standard 6	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22
Standard 7	Standard 7	Standard 7	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23
Standard 8	Standard 8	Standard 8	Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16			

Appendix 2: qPCR Plate Layout

Standard 1	Standard 1	Standard 1	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17
Standard 2	Standard 2	Standard 2	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18
Standard 3	Standard 3	Standard 3	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19
Standard 4	Standard 4	Standard 4	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20
Standard 5	Standard 5	Standard 5	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21
Standard 6	Standard 6	Standard 6	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22
Standard 7	Standard 7	Standard 7	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23
Standard 8	Standard 8	Standard 8	Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	NTC	NTC	NTC