

Application Note

TopCount

AN4002-TC

Calibration, Normalization and IPA™ of the TopCount® NXT™

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Introduction

To enable customers to observe quality assurance, meet high standards for reproducibility from assay to assay, and instill confidence in instrument performance and sample results, Packard Instrument Company has developed a list of unique procedures for its multi-detector TopCount NXT. These procedures will enable users to correctly set up the instrument for various measurements and monitor the performance of the instrument over extended periods of time.

The procedures recommended by Packard to establish and monitor correct instrument performance are:

- **Calibration** – This automatic procedure establishes the correct gain of the system using a special, sealed calibration plate, which has been factory validated for each instrument.
- **Normalization** – The TopCount NXT is multidetector instrument that uses two, six or twelve detectors which can differ in detection efficiency. Normalization ensures consistent detection efficiency and uniformity among detectors.
- **IPA (Instrument Performance Assessment)** – This optional feature monitors five different performance parameters to help ensure reproducible results from the TopCount NXT. When regularly performed, IPA can alert the user of impending problems before they actually affect sample results.

The following discussion describes how to correctly perform each of these procedures, and how to interpret the results.

Calibration

TopCount NXT should be calibrated every week to ensure consistent performance according to factory specifications. **Although the detectors and electronics of the instrument are manufactured under the highest quality standards, subtle changes in performance may occur over time as components age.**

The calibration procedure is easy to initiate; once it begins, it proceeds automatically. When calibration is finished, a calibration report is automatically printed for records. After each successful calibration, the instrument is restored to its factory-specified gain for each detector; it is not necessary to re-establish normalization factors, crosstalk-reduction factors, or quench correction curves. These factors, and quench correction curves need changing only if there has been a change in the assay procedure itself, or if electronic detector components have been replaced.

Each TopCount NXT is shipped with at least one Cal/Norm (Calibration/Normalization) plate. TopCount NXT 9904V and 9912V models are supplied with Cal/Norm plates for both the 96- and 24-well formats. All other models are supplied with only the 96-well Cal/Norm plate. For HTS models, which count either 96- or 384-well microplates, the 96-well Cal/Norm plate is used to calibrate for both the 96- and 384-well applications.



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Each Cal/Norm plate has a unique serial number, the last four digits of which appear on the bar code label on the right side of the plate. Only the Cal/Norm plate(s) initially supplied with the instrument may be used to calibrate that specific instrument. If a Cal/Norm plate(s) is lost or misplaced, validated replacements are available from Packard. Cal/Norm plates must be stored in the dark, in a cool (below 22° C), dry place to ensure best performance.

Cal/Norm plates contain specified amounts of ³H and ¹⁴C in a stable, solid form (no liquid), and these sources are located in specified wells within the plate. The Cal/Norm plate may also be used for normalization under certain conditions, and IPA (See Normalization Section).

Calibration and IPA should always be performed with the counting chamber temperature set at 19° C to ensure run-to-run reproducibility.

Defining and Running a Calibration Assay Without IPA

1. Use the Assay Wizard to define an assay for calibration. Only two screens are required to define the calibration assay, and these are shown as Figures 1a and 1b. Place the Cal/Norm plate in the plate conveyor of the TopCount, and select manual plate loading.
2. Select the calibration assay from the Instrument Control screen.
3. Start the assay.
4. A successful calibration will produce a calibration report such as Figure 2.

A successful Calibration report will show that all detectors have been calibrated (see Figure 2.). If the report shows that not all detectors have been calibrated, repeat the procedure. If the second attempt fails to show that all

detectors have calibrated, the local Packard Service representative should be contacted.

Defining and Running a Calibration Assay With IPA

Calibration is performed as a normal part of the IPA procedure. If an instrument is configured with IPA, and the user wishes to perform the complete IPA procedure, he or she should follow the instructions in this application note, under the section entitled Defining and Running IPA. (See page 5).

Normalization

Detector normalization on the TopCount NXT is performed independently for each assay (protocol). It does not use a separate Assay Definition (protocol) as do Calibration and IPA. Independent detector normalization ensures that the detector counting efficiency is normalized for the microplate, radionuclide and unique chemistry characteristics of each assay. **Normalization must be performed for a newly defined assay before quench curves are counted and stored, and before unknown samples are counted. Normalization or re-normalization should be performed at the same counting chamber temperature that will be used for counting standards and unknowns within the assay.**

The prompt for normalization Factors ($\sqrt{}$) is found in Assay Wizard Step 8 or 9 (See Figures 3. and 4.), depending on whether the assay is CPM or DPM. Once the normalization has been successfully completed, the prompt will no longer appear. **Under most conditions, it is not necessary to re-normalize an assay**, as long as the parameters of the assay have remained constant, and provided that no major changes have been made to the TopCount hardware. However, to ensure stability of the normalized assays, Packard recommends calibration every week.

For most assays it is necessary to prepare only a single normalization sample (single quench level) for normalizing 24-, 96-, or 384-well formats.



Figure 1a.

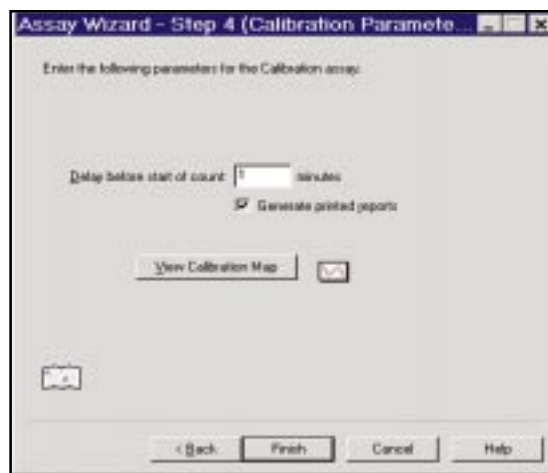


Figure 1b.

Assay Wizard setup screens for defining a calibration assay

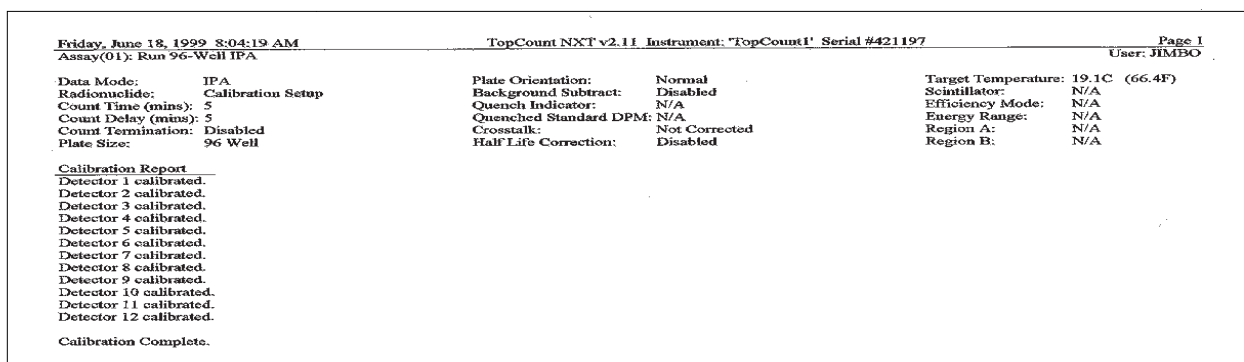


Figure 2.

Calibration printout from a model 9912V VariPlate system indicating the successful calibration of all detectors used to count 96-well plates.



Figure 3.



Figure 4.

Single Quench Level Normalization for Radioisotopic Samples, CPM or DPM

1. Define a new assay using the Assay Wizard, and stop at Step 8 for CPM assays, or Step 9 for DPM assays, to normalize before mapping the assay. [Note: Normalization may also take place after the assay setup is finished.] Before normalizing begins, **the 2% Sigma count terminator in Assay Step 5, under Advanced Count Options, must be set at 0.5%, and the time terminator in Step 5 must be set at a value that will ensure that 160,000 total counts are accumulated for each detector.** (See Figures 5 and 6). If the normalization sample that is prepared in step 2. contains 2,000 CPM, the time terminator should be set for at least 80 minutes. If it contains 25,000 CPM, the time terminator should be set for at least 6.4 minutes (CPM * Time = Total Accumulated Counts). Setting the time and count terminators as indicated above ensures that each detector counts the normalization sample long

enough to accumulate 160,000 counts, which equals a two sigma error of 0.5%. This will provide the best overall normalization for the detectors. Accumulating fewer counts will degrade the normalization, and may result in greater errors among samples.

2. Prepare a normalization sample that matches the chemistry of the unknown samples as much as possible. **The count rate of the normalization sample must be at least 2,000 CPM.** And, the count rate of the normalization sample should be within the mid range of the assay or close to where the most important count rates of the assay will be. Higher count rates of up to 25,000 CPM will ensure faster normalization, especially for 12-detector instruments.
3. Add the normalization sample to position A10 for the 96-well format, or position A20 for the 384-well format. For the 24-well format, add two

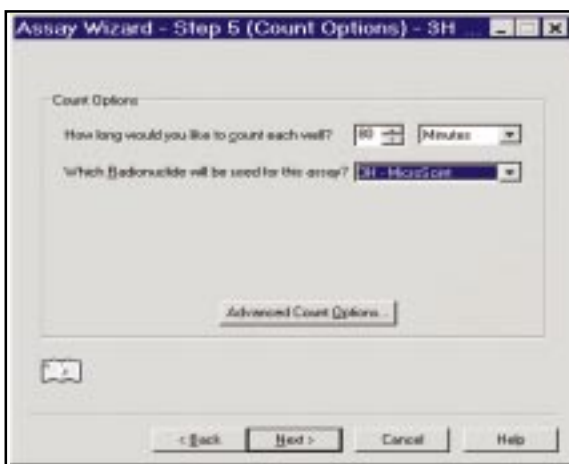


Figure 5.

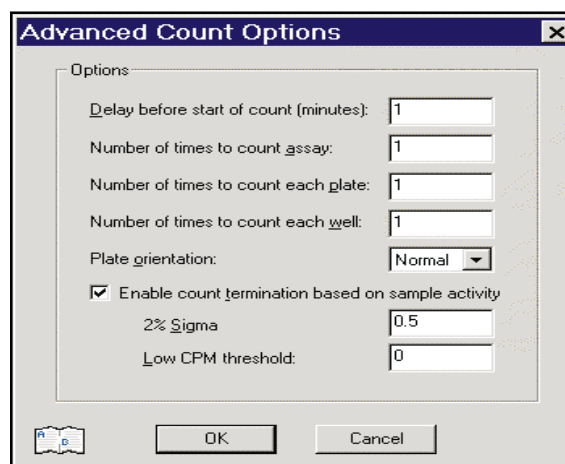


Figure 6.

identical (less than 1% difference) samples to positions A5 and A6. [Note: The same type of microplate, chemistry and radionuclide used for the normalization sample should be used for unknown samples.] Seal the plate with the appropriate TopSeal™.

4. Load the sample plate that contains the normalization sample onto the plate conveyor and select the assay for which the detectors are to be normalized from the Instrument Control screen to start counting the normalization plate.
5. When the instrument completes the normalization procedure, a printed report will indicate whether or not the normalization was successful (See Figure 7).
6. If the normalization was successful, proceed with counting the quench samples or unknown samples, depending on the type of assay that has been defined. If any of the detectors failed to normalize, repeat step 4.

Normalization With the Cal/Norm Plate

For swipe tests, and some other ^3H and $^3\text{H}/^{14}\text{C}$ dual label assays, the factory-provided Cal/Norm plate may be used for single quench level normalization of the instrument, eliminating the need to prepare normalization samples. However, using the Cal/Norm plate for normalization is not recommended for the more demanding requirements of SPA, especially when using ^{125}I . The Cal/Norm plate should be rotated 180 degrees to normalize the instrument, such that well H-12 is where A-1 would normally be positioned. The user should proceed with step 4 from the single quench level normalization procedure.

Multiple Quench Level Normalization for Radioisotopic Samples, CPM or DPM

Sometimes it is preferable to normalize over the entire

quench range covered by the quench curve. This type of normalization may provide tighter normalization for heavily quenched samples, depending on the individual assay.

The multiple-quench level normalization procedure is identical to the single quench level normalization, with the exception of step 3. For multiple-quench normalization, as many as eight samples may be used in column 10 for 96-well, or eight samples in column 20 for 384-well formats. For the 24-well format, up to four each of normalization samples may be used in columns five and six. [Note: The quench range for the multiple-quench normalization must be the same as for the quench curve intended to be used.]

	96-well	384-well	24-well
Least quenched sample	A-10	A-20	A-5, A-6
Next quenched sample	B-10	B-20	B-5, B-6
Next quenched sample	C-10	C-20	C-5, C-6
Most quenched sample	H-10	H-20	D-5, D-6

Once the samples have been placed in the wells for multiple quench normalization, step 4 from the previous procedure should be followed.

Normalization for Luminescence Counting

Detectors must also be normalized for counting luminescence samples, and the procedure is similar to the single quench level normalization used for radioisotopic samples. Packard recommends use of the SPC (Single Photon Counting) Plate Kit (part number 7001129) to ensure that a reproducible luminescence normalization source. However, if the luminescence signal is extremely stable (half-life of more than two hours), one can use his or her own chemistry for luminescence normalization.

Using either the SPC Plate, or your own luminescence chemistry, proceed with the normalization procedure as it is described for the single quench level radioisotopic

Friday, July 16, 1999 8:21:16 AM		TopCount NXT v2.11 Instrument: 'TopCount1' Serial #415999		Page 1
Assay(40): In-Plate Cell Counting Assay				User: DEFAULT
Data Mode:	CPM	Plate Orientation:	Normal	Target Temperature:
Radionuclide:	³ H - MicroScint	Background Subtract:	Disabled	Scintillator:
Count Time (mins):	10	Quench Indicator:	N/A	Efficiency Mode:
Count Delay (mins):	1	Quenched Standard DPM:	N/A	Energy Range:
Count Termination:	Enabled	Crosstalk:	Not Corrected	Region A:
Plate Size:	96 Well	Half Life Correction:	Disabled	Region B:
				2.90 - 35.00
				2.90 - 256.00
Detector Status				
1:	Successful Normalization			
2:	Successful Normalization			
3:	Successful Normalization			
4:	Successful Normalization			
5:	Successful Normalization			
6:	Successful Normalization			
7:	Successful Normalization			
8:	Successful Normalization			
9:	Successful Normalization			
10:	Successful Normalization			
11:	Successful Normalization			
12:	Successful Normalization			

Figure 7.

Normalization printout for the detectors used for 96-well plate counting in a 9912V VariPlate instrument

normalization. Be sure to reduce the sample counting time to about 10 seconds, since the SPC plate produces very high count rates. Also, you should set the delay-before-counting to 25 minutes, to ensure that there is no phosphorescence from the plate.

Normalization for Dual Label Counting

Dual label assays should be normalized using the lower energy radionuclide. For instance, when using ³H/¹⁴C, use ³H to perform the normalization. Then proceed with the normalization as described in previous sections for single quench or multiple quench normalization.

IPA (Instrument Performance Assessment)

Introduction

The IPA feature is designed to help evaluate the performance of the TopCount NXT Microplate Scintillation and Luminescence Counter. IPA measures and stores results for five performance parameters, so each parameter can be monitored for any changes in performance. IPA can alert users (and Packard Service Representatives) of impending performance problems before they affect results. Accordingly, it is recommended that the IPA assay be routinely run, preferably once each week. If this is done, a TopCount NXT's performance can be tracked against historical data. IPA is also useful to help ensure GLP compliance, because it provides proof of the TopCount NXT's performance.

Defining and Running IPA

Define an IPA assay in either the Assay Wizard or Assay Definition application. Assay Wizard is recommended as the easiest setup method (See Figures 8a and 8b).

1. Define a unique Assay Name and number.
2. Select IPA as the Assay Type.

3. Select Plate Type. Depending on the configuration of the TopCount NXT, a 96-well, or either 24- or 96-well plate may be selected. *Note: IPA for the 96-well also applies to the 384-well format, because the same detectors are used for both the 96- and 384-well applications.*
4. Enter description (optional).
5. Click on Next.
6. Select the number of times to count IPA; the number of IPA runs to perform.

[Note: The calibration procedure is performed only once during an unattended IPA run, regardless of the number of times IPA count runs are selected. For routine IPA runs, it is recommended that the default number of times be used to count IPA at 1. This way, each time IPA is run, it will be a complete run, including calibration and all of the other IPA measurements. Calibration of the TopCount NXT should occur on a weekly basis.]

7. To view the IPA plate map, select View IPA Map. The 96-well map will look like the Example in Figure 9.
8. Load the Cal/Norm plate and begin counting IPA. *[Note: An IPA run may be initiated automatically if a bar code label on the Cal/Norm plate is used, or the IPA assay may be manually initialized from the Instrument Control screen. Instrument calibration may also be performed separately by using the Calibration assay setup and the Cal/Norm plate. However, IPA will be performed only if the IPA assay is run.]*

During an IPA run, calibration is performed first, followed by counting all of the mapped sample locations to measure the remaining IPA parameters. When the IPA run has



Figure 8a.

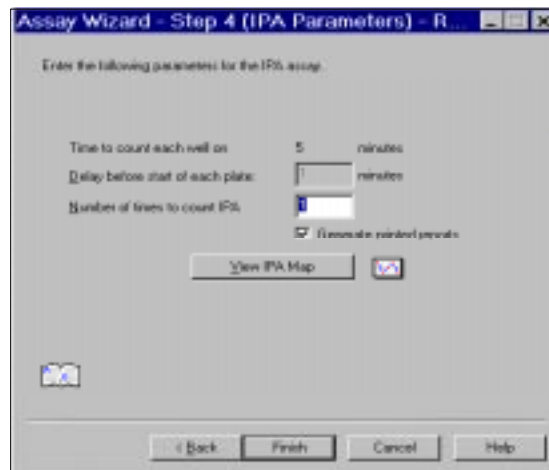


Figure 8b.

Assay Wizard setup for running IPA

finished, all IPA data will be added to the IPA tables and charts, and a printout will show if the instrument passed all test criteria.

Upon installation, and after manually resetting IPA baselines, **three complete IPA runs are required to establish new baselines.** Once established, the new baselines will be used to determine performance of all subsequent IPA runs. To reset the baselines at any time, the Action/Reset Baseline in the IPA Viewer toolbar should be selected. Resetting the baseline does not delete IPA data; it simply relocates the baseline using the average of the three most recent IPA runs.

Using The IPA Viewer

The IPA Viewer (Figure 10.) enables the user to recall, view and print performance tables and charts for each of the IPA parameters below:

- Background CPM uniformity for each detector
- Alignment uniformity for all detectors (96-well

- only)
- High voltage (calibration) uniformity for each detector
- ³H CPM (efficiency) repeatability
- ³H tSIS (gain) repeatability

To open the IPA Viewer, double-click on the IPA Viewer icon on your desktop screen (or wherever you chose to place it).

To examine a desired IPA parameter, simply select its button on the toolbar. From left to right, the buttons represent: background uniformity, detector alignment uniformity, high voltage uniformity, ³H CPM uniformity and ³H tSIS uniformity. When either background uniformity or high voltage uniformity is selected, the desired detector must also be selected.

Any chart may be printed simply by selecting the printer button while viewing the chart. A printout of the



Figure 9.

IPA plate map for the 96-well IPA procedure

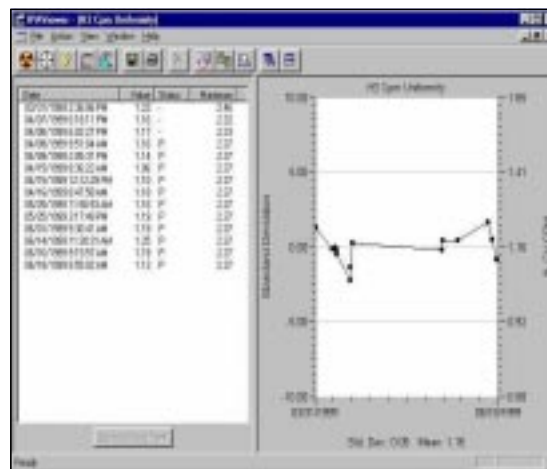


Figure 10.

data table will accompany the chart printout.

A chart's scale may be changed within the View drop-down menu.

Selected data points can also be deleted from the table and corresponding chart. To delete a point, move the mouse pointer to the point and select it with a single click of the left mouse button. To delete the selected point, a simple click on the Remove Data Point button is all that is needed. *[Note: When any point is deleted, it will be completely removed, and cannot be reinstated to the table or chart. To prevent unauthorized deletion of IPA points, there is a password protection feature on the TopCount NXT that prevents unauthorized users from accessing the IPA viewer.]*

Interpreting IPA Results

The following are the criteria used to evaluate IPA count results. Evaluation can result in two different types of warnings: system-wide, or per detector.

- **Background:** If any detector background value is greater than three (3) times the baseline value, and greater than the instrument specifications (62 CPM for the 96-well format, and 100 CPM for the 24-well format), a per-detector warning message will be generated. This is very useful to reveal detector contamination.
- **Detector alignment (96-well only):** If the background-corrected CPM of any non-active* well adjacent to a ¹⁴C-containing well, is greater than 0.3% of the ¹⁴C-containing well's CPM, a system-wide warning is generated. This test measures how accurately each detector is positioned over the wells. *contains no radioactivity

- **High voltage:** If the high voltage for any detector deviates from the baseline by more than +/- 5%, a per-detector warning message will be generated. This is a test to measure the gain stability of each detector. Frequent changes here may signal an impending PMT problem.
- **³H CPM uniformity:** %CV (Percent Coefficient of Variation) is greater than two-times the baseline, a system-wide warning message will be generated. This test measures the repeatability of ³H counting. Extraordinary changes in the count rates from any of the detectors may indicate a PMT problem.
- **tSIS uniformity:** tSIS (Transformed Spectral Index of the Sample) is a quench indicating parameter. If the tSIS %CV is greater than two-times the baseline, a system-wide warning message will be generated. This test measures the repeatability of the quench indicating parameter, tSIS, for ³H. This test, and the CPM uniformity test measure the stability of count rate and spectrum end point determinations.

A successful IPA run will indicate that all parameters have passed the IPA criteria, and that there are no impending problems with the instrument (See Figure 11). *[Note: the printed calibration and IPA results should be stored in a safe place for future reference.]*

IPA parameters normally remain within the specified limits and maintain statistically consistent performance over time. However, when a failure occurs, the entire IPA run should be repeated, and the results should be compared with the results that generated the failure warning, and

Instrument Performance Parameter		Status	Value	Minimum Value	Maximum Value
Detector 1 Background	Pass	CPM = 2.80	--	15.78	
Detector 2 Background	Pass	CPM = 7.14	--	30.64	
Detector 3 Background	Pass	CPM = 15.18	--	52.78	
Detector 4 Background	Pass	CPM = 8.62	--	44.50	
Detector 5 Background	Pass	CPM = 3.86	--	13.66	
Detector 6 Background	Pass	CPM = 23.80	--	86.18	
Detector 7 Background	Pass	CPM = 16.74	--	70.16	
Detector 8 Background	Pass	CPM = 4.84	--	20.08	
Detector 9 Background	Pass	CPM = 11.08	--	45.38	
Detector 10 Background	Pass	CPM = 11.32	--	27.34	
Detector 11 Background	Pass	CPM = 9.46	--	46.44	
Detector 12 Background	Pass	CPM = 9.78	--	42.10	
System Detector Alignment	Pass	%Crosstalk = 0.06	--	0.30	
Detector 1 High Voltage	Pass	HV Dao = 1088.57	1034.48	1143.37	
Detector 2 High Voltage	Pass	HV Dao = 1078.43	1026.37	1134.41	
Detector 3 High Voltage	Pass	HV Dao = 1079.31	1026.38	1134.64	
Detector 4 High Voltage	Pass	HV Dao = 1096.43	1041.19	1150.79	
Detector 5 High Voltage	Pass	HV Dao = 1064.91	1011.40	1117.87	
Detector 6 High Voltage	Pass	HV Dao = 1097.02	1041.72	1151.38	
Detector 7 High Voltage	Pass	HV Dao = 967.47	923.03	1020.19	
Detector 8 High Voltage	Pass	HV Dao = 1039.33	990.11	1094.33	
Detector 9 High Voltage	Pass	HV Dao = 1001.27	953.08	1054.40	
Detector 10 High Voltage	Pass	HV Dao = 1066.08	1015.17	1122.03	
Detector 11 High Voltage	Pass	HV Dao = 962.40	915.42	1011.78	
Detector 12 High Voltage	Pass	HV Dao = 967.47	921.35	1018.34	
System H3 Cpm Uniformity	Pass	%CV = 1.13	--	2.37	
System H3 tSIS Uniformity	Pass	%CV = 4.36	--	8.78	

Figure 11.

IPA printout example for the model 9912V VariPlate instrument. The example shows IPA results for the six detectors which are used for counting 96-well microplates.

with previous IPA runs.

Also, if repeated failure warnings, or trends (up or down) for any IPA parameter are observed, the Packard Service Engineer should be notified. The IPA parameters normally remain within specified limits, causing instrument performance to be statistically level over time. Deviations from normal behavior of the IPA should be reported.

Summary

Users can ensure the highly reproducible performance of the TopCount NXT if they simply follow the procedures for calibration and normalization. Regular and frequent (weekly) calibration is the key to maintaining stable performance for all assays. IPA provides a unique and extremely valuable tool for monitoring the performance of the instrument, for compliance with GLP, and to ensure against potential problems before they affect results.

In addition to the above procedures, it is necessary for some assays to also correct for background, isotopic crosstalk and variable quench. These procedures are assay- (protocol) specific, and are unique for each assay. For detailed discussions of these procedures, consult the TopCount Operation or Reference manual and TopCount Topics # 001-005.