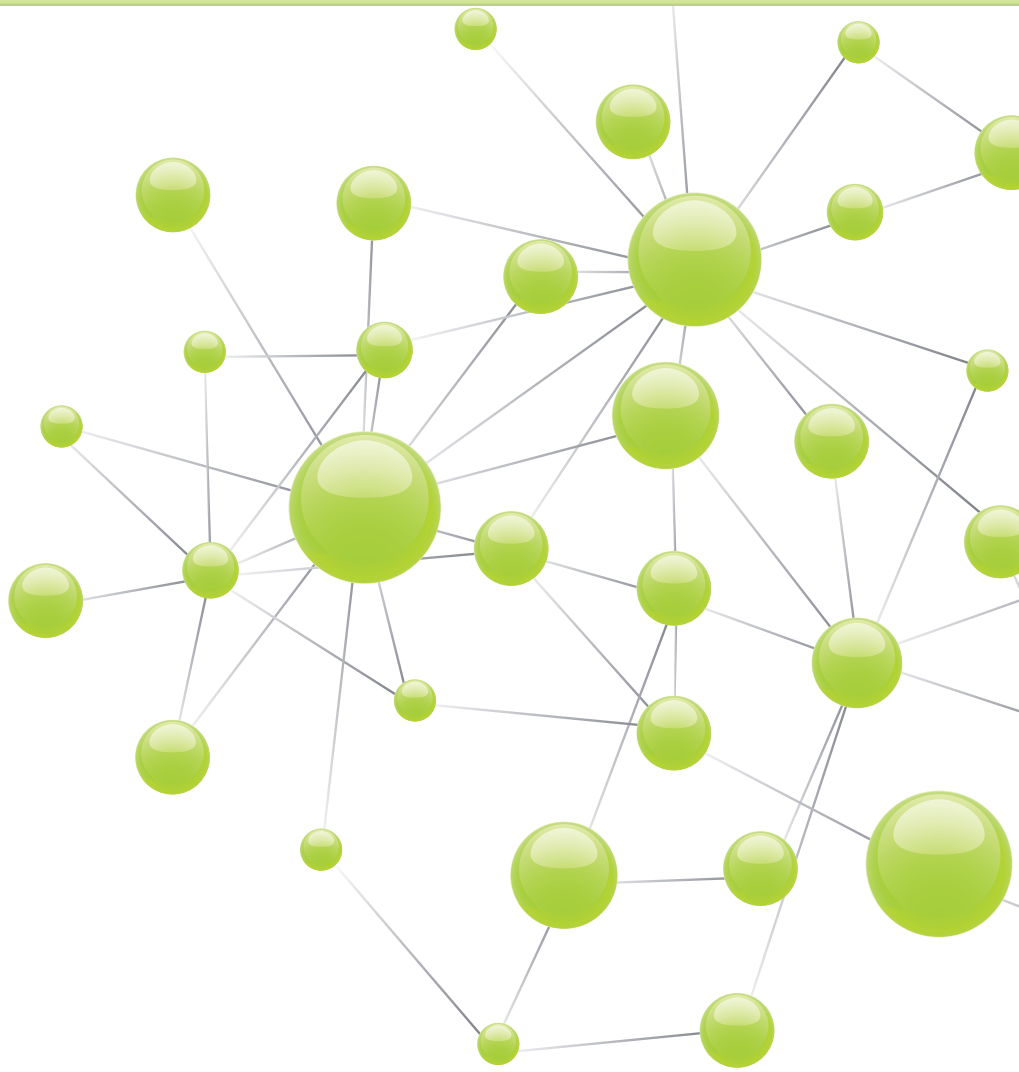
nanoString™
TECHNOLOGIES



nCounter® CNV

# nCounter®

# Data Analysis Guidelines *for* Copy Number Variation (CNV)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Molecules That Count®

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MAN-C0014-02

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# PREFACE

## Conventions Used

The following conventions are used throughout this manual and are described below for your reference:

### Note Types

Special font formatting is used in this manual. Such formatting conventions are used in specific instances as described below:

**TIP** Information contained in a Tip may offer helpful suggestions, alternative procedures, methods and/or shortcuts.

**NOTE** This note type emphasizes general information.

**IMPORTANT**  This note type presents essential content indicating that the potential exists for assay failure, diminished data quality, and/or a loss of data if the information presented is ignored.

**WARNING**  This note type indicates that a potential hazard to your personal safety, or the potential for equipment damage exists.

**BOLD** When appearing in text or in a procedure, the bold text serves to highlight a specific button, key stroke, or menu option available.

- **Bold** text may appear elsewhere to highlight important text or terms.
- **Green** text is used to help the reader identify active hyperlinks.

**ITALICS** Used to emphasize an important word or expression within the text.

- Formatting of a book title, journal, or other documentation.
- Used to indicate the special or unusual meaning of a word or phrase.

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# 1 Introduction

The nCounter® Custom Copy Number Variation (CNV) Assay utilizes NanoString's unique direct and multiplexed detection of nucleic acids in solution to generate estimates of copy number variation for hundreds of loci in a single reaction. Each NanoString Reporter and Capture Probe pair is complementary to ~100 nt of contiguous genomic DNA sequence at a user-specified locus. Genomic DNA is fragmented into small pieces (200-800 bp) and denatured to produce single strands. The Custom CNV CodeSet is then hybridized to the fragmented, denatured DNA sample in a single multiplexed reaction (up to 800 genomic loci per CodeSet). Hybridized DNA-CodeSet complexes are purified by the fully automated nCounter® Prep Station, and Reporters are counted by the nCounter® Digital Analyzer.

The results of an nCounter® Custom CNV Assay experiment are compiled and displayed using the CNV Collector Tool software included with the assay kit. Detailed instructions for using this tool are in the **CNV Collector Tool User Manual** provided with the software.

The following Data Analysis Guidelines for CNV are intended as a supplement to the **nCounter® CNV Collector Tool User Manual**. They provide instructions and additional information for those who wish to do further QC and/or data manipulations with the data output from the CNV Collector Tool, such as:

- **Modify the normalization method**
- **Apply additional assay quality control metrics**
- **Change reference samples within a single data set**
- **Use reference samples with known copy numbers that differ from 2**

## nCounter Custom CNV Data Output

### Raw Data

The basic output of the Custom CNV Assay is a spreadsheet containing the CodeSet probe identifiers, sample identifiers, and the digital 'counts' recorded for each probe in each sample. This file, referred to as a Reporter Code Count (RCC) file, can be uploaded to the nCounter CNV Collector Tool for automated normalization and analysis.

**FIGURE 1.1:** Report Code Count (RCC) file

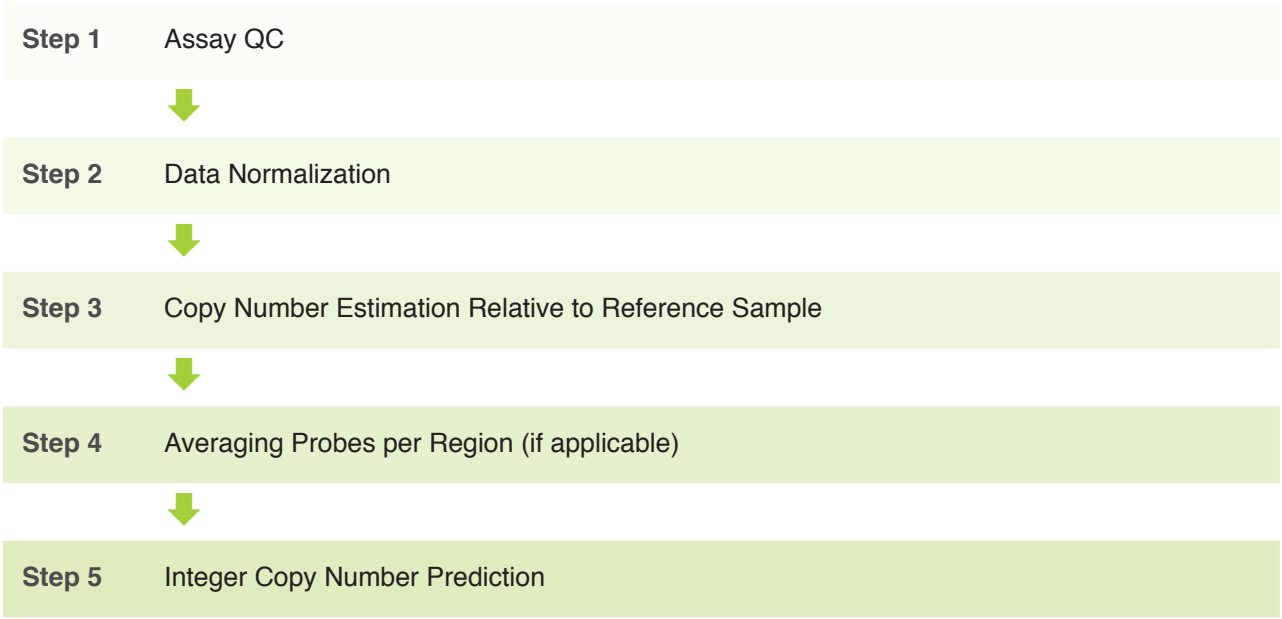
| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 19 | **Reporter Counts** | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 20 | *Code Class* | *Name* | *Accession* | | | | | | |
| 21 | Positive | POS_A(128) | ERCC_00117.1 | 21918 | 25182 | 20426 | 26194 | 25293 | 22480 |
| 22 | Positive | POS_B(32) | ERCC_00112.1 | 6608 | 7521 | 4640 | 6107 | 6070 | 5367 |
| 23 | Positive | POS_C(8) | ERCC_00002.1 | 1405 | 1627 | 1351 | 1623 | 1637 | 1464 |
| 24 | Positive | POS_D(2) | ERCC_00092.1 | 366 | 429 | 284 | 406 | 402 | 331 |
| 25 | Positive | POS_E(0.5) | ERCC_00035.1 | 139 | 161 | 242 | 261 | 270 | 241 |
| 26 | Positive | POS_F(0.125) | ERCC_00034.1 | 65 | 72 | 87 | 80 | 79 | 80 |
| 27 | Negative | NEG_A(0) | ERCC_00096.1 | 0 | 1 | 6 | 8 | 14 | 7 |
| 28 | Negative | NEG_B(0) | ERCC_00041.1 | 3 | 7 | 9 | 11 | 13 | 15 |
| 29 | Negative | NEG_C(0) | ERCC_00019.1 | 8 | 6 | 9 | 10 | 9 | 10 |
| 30 | Negative | NEG_D(0) | ERCC_00076.1 | 3 | 2 | 16 | 20 | 15 | 12 |

## Code Classes

| | |
|---|---|
| **1. POSITIVE** | nCounter Custom CNV CodeSets contains 6 positive dsDNA control probes, each targeting a unique DNA sequence present in every assay. The concentrations of DNA target range from 0.125 fM to 128 fM in the hybridization reaction. |
| **2. NEGATIVE** | nCounter Custom CNV CodeSets contain 8 negative control probes, for which there is no DNA target present in the hybridization reaction. These probes monitor the nonspecific, or background, counts for every assay. |
| **3. INVARIANT** | Each CodeSet contains a set of 10 probes (INV) designed to autosomal genomic regions predicted not to contain common CNVs. |
| **4. RESTRICTION SITE** | Custom CNV CodeSets contain four control probes to monitor the efficiency of the DNA fragmentation and denaturation steps of the CNV Assay Protocol. Probes A and B are designed to a DNA sequence containing an AluI restriction site, and will return low count when the AluI fragmentation is working correctly. Probes C and D are designed to sequences that lack an AluI restriction site, and serve as controls for the presence of target DNA in the sample preparation step. When used according to the assay manual, these controls help identify problems in restriction enzyme fragmentation and denaturation steps of the assay. |
| **5. ENDOGENOUS** | The Custom CNV Assay probes specified by the user, designed to specific regions of the genome. |

## Basic CNV Data Analysis Workflow

**FIGURE 1.2:** CNV Data Analysis Workflow



## Assay Quality Control Check

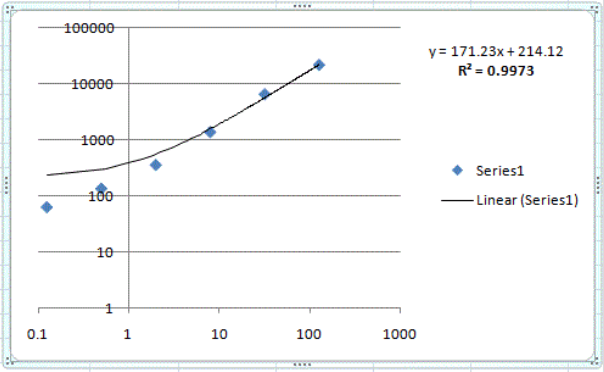
When used according to the assay manual, each Custom CNV Assay contains controls which monitor hybridization efficiency, sample DNA fragmentation denaturation, and sample DNA input amount. Before continuing with CNV analysis, first look at the Raw Data output file from the CNV Collector to gauge the performance of the assay.

### Positive Controls

The positive control (POS) DNA targets are added in a linear titration to each codeset to generate a standard curve. The final concentration in the hybridization of each target (in fM) is indicated in parentheses next to the POS probe identifier. To check the linearity of the POS control standard curve, insert a new column in the Raw Data spreadsheet to the left of the first assay data column. Add the POS target concentrations to the first 6 rows of this new column. Then create a scatter plot of the target concentration vs. raw data for each target (**Figure 1.3**).

**FIGURE 1.3:** Positive Controls

| | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | **Reporter Counts** | | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 | |
| 20 | *Code Class* | *Name* | *Accession* | | | | | | | | |
| 21 | Positive | POS_A(128) | ERCC_00117.1 | 128 | 21918 | | | | | | |
| 22 | Positive | POS_B(32) | ERCC_00112.1 | 32 | 6608 | | | | | | |
| 23 | Positive | POS_C(8) | ERCC_00002.1 | 8 | 1405 | | | | | | |
| 24 | Positive | POS_D(2) | ERCC_00092.1 | 2 | 366 | | | | | | |
| 25 | Positive | POS_E(0.5) | ERCC_00035.1 | 0.5 | 139 | | | | | | |
| 26 | Positive | POS_F(0.125) | ERCC_00034.1 | 0.125 | 65 | | | | | | |
| 27 | Negative | NEG_A(0) | ERCC_00096.1 | | 0 | | | | | | |
| 28 | Negative | NEG_B(0) | ERCC_00041.1 | | 3 | | | | | | |
| 29 | Negative | NEG_C(0) | ERCC_00019.1 | | 8 | | | | | | |
| 30 | Negative | NEG_D(0) | ERCC_00076.1 | | 3 | | | | | | |
| 31 | Negative | NEG_E(0) | ERCC_00098.1 | | 5 | | | | | | |
| 32 | Negative | NEG_F(0) | ERCC_00126.1 | | 1 | | | | | | |
| 33 | Negative | NEG_G(0) | ERCC_00144.1 | | 4 | | | | | | |
| 34 | Negative | NEG_H(0) | ERCC_00154.1 | | 0 | | | | | | |




In the scatter plot shown in Figure 1.3, the axes are represented in logarithmic scale and the linear regression ($R^2$) is shown in the inset. The $R^2$ value for the POS control probes should be > 0.95.

### Restriction Fragmentation Controls

The Custom CNV Assay Kit comes with a set of four DNA controls that, when added to your genomic DNA sample prior to fragmentation, will monitor the efficiency of enzymatic digestion and heat denaturation.

The DNA targets for probes labeled RESTRICTIONSITE+A and RESTRICTIONSITE+B contain an AluI restriction site such that, after complete digestion, the target site will be cleaved by the enzyme and low probe count will be observed. The DNA targets for probes labeled RESTRICTIONSITE-C and RESTRICTIONSITE-D do not contain AluI sites, and will generate probe counts even in the absence of fragmentation. These targets will serve as controls for proper addition of the control DNA to the sample and proper heat denaturation. If the DNA sample is not denatured prior to hybridization, you will observe low counts (generally < 200) for RESTRICTIONSITE-C and RESTRICTIONSITE-D probes.

When the genomic DNA sample is completely digested with AluI enzyme and denatured, you should observe at least a 10-fold difference in counts between RESTRICTIONSITE+ (Probes A and B) and RESTRICTIONSITE- probes (Probes C and D):

**FIGURE 1.4:** Restriction Fragmentation Controls

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 50 | RestrictionSite | _RESTRICTIONSITE+A\|+\|CTL\|AluI+A | _RESTRICTIONSITE | 12 | 15 | 25 | 43 | 41 | 47 |
| 51 | RestrictionSite | _RESTRICTIONSITE+B\|+\|CTL\|AluI+B | _RESTRICTIONSITE | 10 | 15 | 54 | 71 | 83 | 47 |
| 52 | RestrictionSite | _RESTRICTIONSITE-C\|-\|CTL\|AluI-C | _RESTRICTIONSITE | 1255 | 1399 | 1144 | 1331 | 1295 | 1120 |
| 53 | RestrictionSite | _RESTRICTIONSITE-D\|-\|CTL\|AluI-D | _RESTRICTIONSITE | 975 | 1115 | 1336 | 1548 | 1541 | 1305 |

## Normalization to Invariant Probes

Each nCounter Custom CNV CodeSet contains probes designed to invariant regions of 10 autosomes. It is assumed that these 10 regions will represent 2 chromosomal copies in a vast majority of samples analyzed. Therefore, normalizing data to the counts obtained from these 10 invariant probes should correct for any differences in sample to sample genomic DNA input arising from pipetting error or inaccuracies in DNA quantitation. Normalization is performed automatically by the nCounter CNV Collector Tool, but can be done manually as follows:

1. To normalize data to the Invariant probes, calculate the average count value for the 10 INV probes (AVE INV) in the first lane (sample) as shown in **Figure 1.5**:

**FIGURE 1.5:** Calculating the average count value.

| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 19 | Reporter Counts | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 20 | *Code Class* | *Name* | *Accession* | | | | | | |
| 21 | Positive | POS_A(128) | ERCC_00117.1 | 21918 | 25182 | 20426 | 26194 | 25293 | 22480 |
| 22 | Positive | POS_B(32) | ERCC_00112.1 | 6608 | 7521 | 4640 | 6107 | 6070 | 5367 |
| 23 | Positive | POS_C(8) | ERCC_00002.1 | 1405 | 1627 | 1351 | 1623 | 1637 | 1464 |
| 24 | Positive | POS_D(2) | ERCC_00092.1 | 366 | 429 | 284 | 406 | 402 | 331 |
| 25 | Positive | POS_E(0.5) | ERCC_00035.1 | 139 | 161 | 242 | 261 | 270 | 241 |
| 26 | Positive | POS_F(0.125) | ERCC_00034.1 | 65 | 72 | 87 | 80 | 79 | 80 |
| 27 | Negative | NEG_A(0) | ERCC_00096.1 | 0 | 1 | 6 | 8 | 14 | 7 |
| 28 | Negative | NEG_B(0) | ERCC_00041.1 | 3 | 7 | 9 | 11 | 13 | 15 |
| 29 | Negative | NEG_C(0) | ERCC_00019.1 | 8 | 6 | 9 | 10 | 9 | 10 |
| 30 | Negative | NEG_D(0) | ERCC_00076.1 | 3 | 2 | 16 | 20 | 15 | 12 |
| 31 | Negative | NEG_E(0) | ERCC_00098.1 | 5 | 11 | 11 | 7 | 9 | 11 |
| 32 | Negative | NEG_F(0) | ERCC_00126.1 | 1 | 2 | 15 | 13 | 15 | 12 |
| 33 | Negative | NEG_G(0) | ERCC_00144.1 | 4 | 7 | 10 | 8 | 13 | 15 |
| 34 | Negative | NEG_H(0) | ERCC_00154.1 | 0 | 2 | 13 | 14 | 19 | 13 |
| 35 | | | | | | | | | |
| 36 | | | | =AVERAGE(D40:D49) | 485 | 696 | 674 | 758 | 754 |
| 37 | | | average | AVERAGE(number1, [number2], ...) | | | | | |
| 38 | | | **Norm.factor:** | **0.97** | **1.40** | **0.97** | **1.00** | **0.89** | **0.90** |
| 39 | | | | | | | | | |
| 40 | Invariant | _INVCONTROL-1\|+\|Hg18\|chr1:97239055-97239154 | _INVCONTROL | 985 | 632 | 906 | 856 | 880 | 995 |
| 41 | Invariant | _INVCONTROL-10\|+\|Hg18\|chr10:76411622-76411718 | _INVCONTROL | 382 | 226 | 414 | 388 | 435 | 440 |
| 42 | Invariant | _INVCONTROL-16\|+\|Hg18\|chr16:11026537-11026636 | _INVCONTROL | 433 | 356 | 495 | 500 | 575 | 571 |
| 43 | Invariant | _INVCONTROL-17\|+\|Hg18\|chr17:60617472-60617571 | _INVCONTROL | 792 | 530 | 732 | 761 | 813 | 832 |
| 44 | Invariant | _INVCONTROL-19\|+\|Hg18\|chr19:38818850-38818949 | _INVCONTROL | 656 | 464 | 715 | 653 | 702 | 694 |
| 45 | Invariant | _INVCONTROL-2\|+\|Hg18\|chr2:137221998-137222092 | _INVCONTROL | 605 | 414 | 636 | 586 | 739 | 663 |
| 46 | Invariant | _INVCONTROL-20\|+\|Hg18\|chr20:10504898-10504992 | _INVCONTROL | 488 | 320 | 511 | 527 | 566 | 538 |
| 47 | Invariant | _INVCONTROL-22\|+\|Hg18\|chr22:42509372-42509471 | _INVCONTROL | 895 | 637 | 828 | 847 | 991 | 936 |
| 48 | Invariant | _INVCONTROL-5\|+\|Hg18\|chr5:71261236-71261335 | _INVCONTROL | 913 | 690 | 887 | 837 | 956 | 991 |
| 49 | Invariant | _INVCONTROL-7\|+\|Hg18\|chr7:41541153-41541252 | _INVCONTROL | 800 | 577 | 838 | 789 | 919 | 878 |
| 50 | RestrictionSite | _RESTRICTIONSITE+A\|+\|CTL\|AluI+A | _RESTRICTIONSITE | 12 | 15 | 25 | 43 | 41 | 47 |



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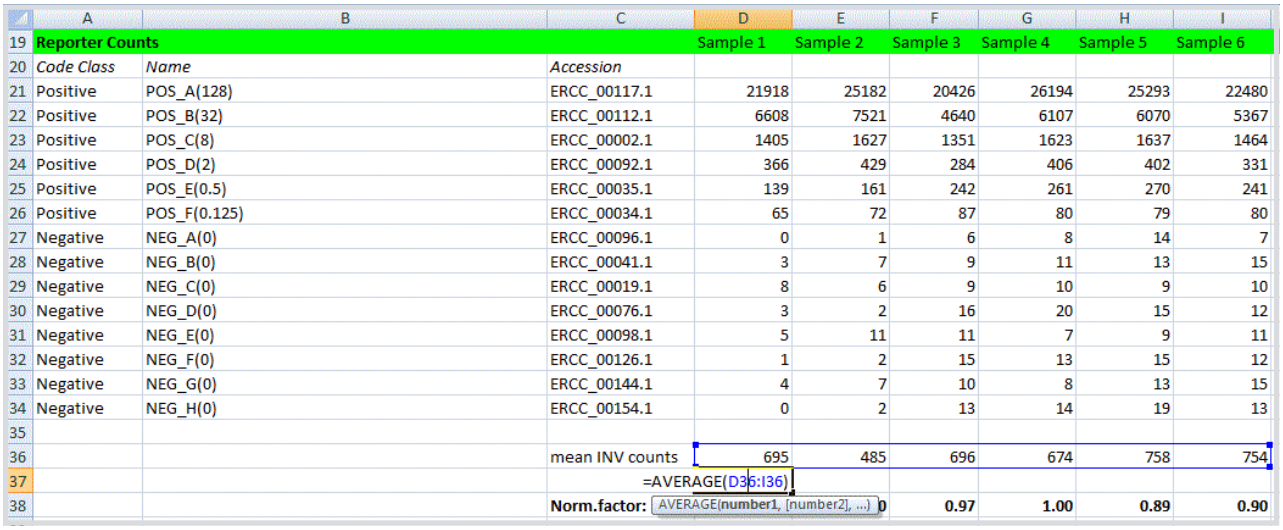
2. Calculate a normalization factor for each assay by first calculating the mean of the average INV count values (mean AVE INV) across all lanes you wish to analyze (**Figure 1.6**):

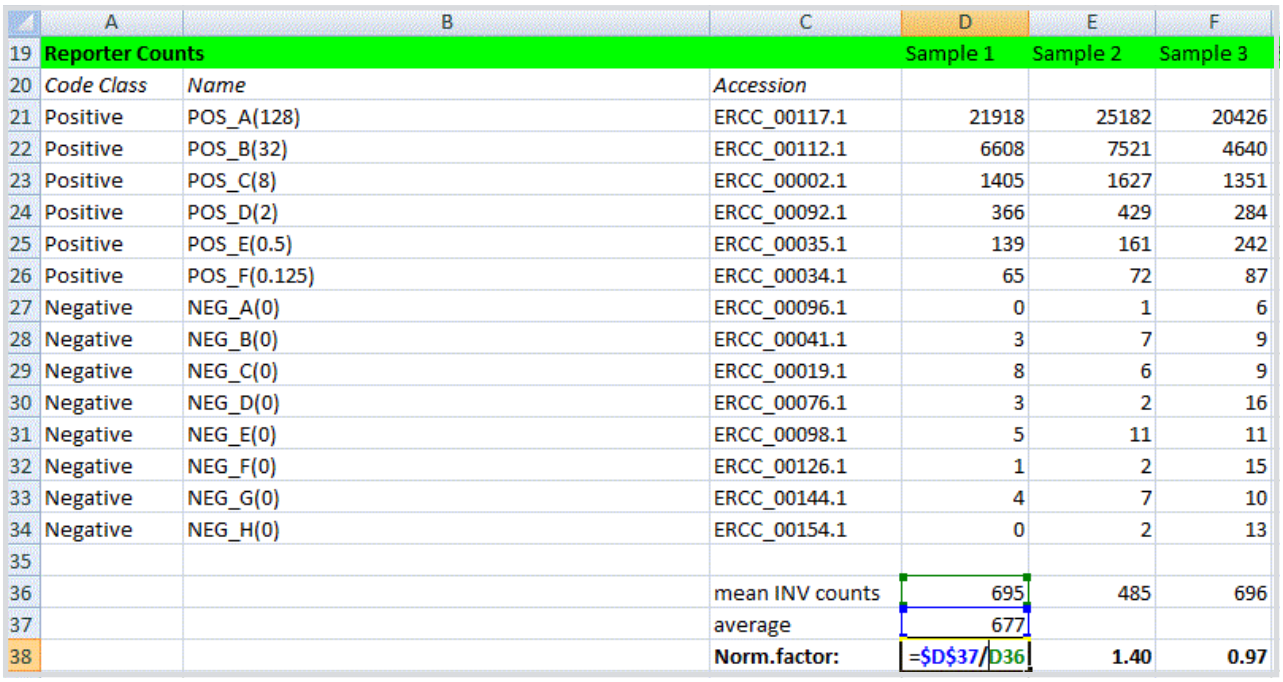
**FIGURE 1.6:** Calculating the mean of the average INV count values.



| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 19 | **Reporter Counts** | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 20 | *Code Class* | *Name* | *Accession* | | | | | | |
| 21 | Positive | POS_A(128) | ERCC_00117.1 | 21918 | 25182 | 20426 | 26194 | 25293 | 22480 |
| 22 | Positive | POS_B(32) | ERCC_00112.1 | 6608 | 7521 | 4640 | 6107 | 6070 | 5367 |
| 23 | Positive | POS_C(8) | ERCC_00002.1 | 1405 | 1627 | 1351 | 1623 | 1637 | 1464 |
| 24 | Positive | POS_D(2) | ERCC_00092.1 | 366 | 429 | 284 | 406 | 402 | 331 |
| 25 | Positive | POS_E(0.5) | ERCC_00035.1 | 139 | 161 | 242 | 261 | 270 | 241 |
| 26 | Positive | POS_F(0.125) | ERCC_00034.1 | 65 | 72 | 87 | 80 | 79 | 80 |
| 27 | Negative | NEG_A(0) | ERCC_00096.1 | 0 | 1 | 6 | 8 | 14 | 7 |
| 28 | Negative | NEG_B(0) | ERCC_00041.1 | 3 | 7 | 9 | 11 | 13 | 15 |
| 29 | Negative | NEG_C(0) | ERCC_00019.1 | 8 | 6 | 9 | 10 | 9 | 10 |
| 30 | Negative | NEG_D(0) | ERCC_00076.1 | 3 | 2 | 16 | 20 | 15 | 12 |
| 31 | Negative | NEG_E(0) | ERCC_00098.1 | 5 | 11 | 11 | 7 | 9 | 11 |
| 32 | Negative | NEG_F(0) | ERCC_00126.1 | 1 | 2 | 15 | 13 | 15 | 12 |
| 33 | Negative | NEG_G(0) | ERCC_00144.1 | 4 | 7 | 10 | 8 | 13 | 15 |
| 34 | Negative | NEG_H(0) | ERCC_00154.1 | 0 | 2 | 13 | 14 | 19 | 13 |
| 35 | | | | | | | | | |
| 36 | | | mean INV counts | 695 | 485 | 696 | 674 | 758 | 754 |
| 37 | | | =AVERAGE(D36:I36) | | | | | | |
| 38 | | | **Norm.factor:** AVERAGE(**number1**, [number2], ...) | **0** | **0.97** | **1.00** | **0.89** | **0.90** | |

To generate a normalization factor, divide the mean average value (mean AVE INV from Step 2) by the average count value (AVE INV from Step 1) for each lane:

**FIGURE 1.7:** Generating a Normalization Factor



| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| 19 | **Reporter Counts** | | | Sample 1 | Sample 2 | Sample 3 |
| 20 | *Code Class* | *Name* | *Accession* | | | |
| 21 | Positive | POS_A(128) | ERCC_00117.1 | 21918 | 25182 | 20426 |
| 22 | Positive | POS_B(32) | ERCC_00112.1 | 6608 | 7521 | 4640 |
| 23 | Positive | POS_C(8) | ERCC_00002.1 | 1405 | 1627 | 1351 |
| 24 | Positive | POS_D(2) | ERCC_00092.1 | 366 | 429 | 284 |
| 25 | Positive | POS_E(0.5) | ERCC_00035.1 | 139 | 161 | 242 |
| 26 | Positive | POS_F(0.125) | ERCC_00034.1 | 65 | 72 | 87 |
| 27 | Negative | NEG_A(0) | ERCC_00096.1 | 0 | 1 | 6 |
| 28 | Negative | NEG_B(0) | ERCC_00041.1 | 3 | 7 | 9 |
| 29 | Negative | NEG_C(0) | ERCC_00019.1 | 8 | 6 | 9 |
| 30 | Negative | NEG_D(0) | ERCC_00076.1 | 3 | 2 | 16 |
| 31 | Negative | NEG_E(0) | ERCC_00098.1 | 5 | 11 | 11 |
| 32 | Negative | NEG_F(0) | ERCC_00126.1 | 1 | 2 | 15 |
| 33 | Negative | NEG_G(0) | ERCC_00144.1 | 4 | 7 | 10 |
| 34 | Negative | NEG_H(0) | ERCC_00154.1 | 0 | 2 | 13 |
| 35 | | | | | | |
| 36 | | | mean INV counts | 695 | 485 | 696 |
| 37 | | | average | 677 | | |
| 38 | | | **Norm.factor:** | =$D$37/D36 | **1.40** | **0.97** |

3. Calculate INV-normalized counts for each probe. On a new sheet, generate normalized counts for each probe in the CodeSet by multiplying the RAW counts for each probe by the normalization factor for the lane as follows:

FIGURE 1.8

| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 21 | **Reporter Counts** | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 22 | *Code Class* | *Name* | *Accession* | | | | | | |
| 23 | Invariant | _INVCONTROL-1\|+\|Hg18\|chr1:97239055-97239154 | _INVCONTROL | ='Raw data example'!D40*'Raw data example'!D$38 | | | | | |
| 24 | Invariant | _INVCONTROL-10\|+\|Hg18\|chr10:76411622-76411718 | _INVCONTROL | 372 | 316 | 403 | 389 | 389 | |
| 25 | Invariant | _INVCONTROL-16\|+\|Hg18\|chr16:11026537-11026636 | _INVCONTROL | 422 | 497 | 481 | 502 | 514 | |
| 26 | Invariant | _INVCONTROL-17\|+\|Hg18\|chr17:60617472-60617571 | _INVCONTROL | 772 | 740 | 712 | 764 | 726 | |
| 27 | Invariant | _INVCONTROL-19\|+\|Hg18\|chr19:38818850-38818949 | _INVCONTROL | 639 | 648 | 695 | 655 | 627 | |
| 28 | Invariant | _INVCONTROL-2\|+\|Hg18\|chr2:137221998-137222092 | _INVCONTROL | 589 | 578 | 618 | 588 | 660 | |
| 29 | Invariant | _INVCONTROL-20\|+\|Hg18\|chr20:10504898-10504992 | _INVCONTROL | 475 | 447 | 497 | 529 | 506 | |
| 30 | Invariant | _INVCONTROL-22\|+\|Hg18\|chr22:42509372-42509471 | _INVCONTROL | 872 | 890 | 805 | 850 | 885 | |
| 31 | Invariant | _INVCONTROL-5\|+\|Hg18\|chr5:71261236-71261335 | _INVCONTROL | 889 | 964 | 862 | 840 | 854 | |
| 32 | Invariant | _INVCONTROL-7\|+\|Hg18\|chr7:41541153-41541252 | _INVCONTROL | 779 | 806 | 815 | 792 | 821 | |
| 33 | Endogenous | probe1\|+\|Hg18\|chr1: *coordinates* | CNV region 1 | 204 | 330 | 225 | 269 | 303 | |
| 34 | Endogenous | probe2\|+\|Hg18\|chr8: *coordinates* | CNV region 2 | 456 | 711 | 155 | 225 | 237 | |
| 35 | Endogenous | probe3\|+\|Hg18\|chr10: *coordinates* | CNV region 3 | 399 | 609 | 284 | 377 | 415 | |
| 36 | Endogenous | probe4\|+\|Hg18\|chr12: *coordinates* | CNV region 4 | 480 | 779 | 232 | 317 | 320 | |
| 37 | Endogenous | probe5\|+\|Hg18\|chrX:111441962-111442061 | CNV region 5 | 444 | 265 | 312 | 207 | 469 | |

Raw data example | INV norm example | CN estimate example | Integer Copy Number example

For copy number analysis, it is not necessary to normalize the data for POS, NEG or Restriction Site controls. For simplicity, these probes can be left out of the INV normalization spreadsheet. The INV-normalization procedure should be carried out on the Invariant control and Endogenous code classes.

**CAUTION:** Copy number data generated by the Custom CNV Assay can be negatively affected when DNA input amounts are too low, at which point sampling error can introduce unacceptably high levels of variation in the data. NanoString recommends a minimum of 100 counts for the average of the 10 Invariant control probes in the INV-normalized data set to ensure reliable copy number estimation. This is particularly important for the reference sample(s), since accurate copy number calculations depend upon high quality reference sample data. A poor quality reference sample will adversely affect copy number calls for all samples. A poor quality test sample will result in unreliable copy number call for that sample alone. In general for purified genomic DNA, free of RNA contamination, 100 INV-normalized counts will correspond to ≥ 100 ng genomic DNA.

## Calculating Copy Number Estimates

### Reference Sample Selection

The basic data analysis strategy for determining copy numbers with the nCounter Custom CNV Assay is to calculate a copy number estimate for each probe relative to a reference sample (or samples). Each probe in the Custom CNV CodeSet is a unique sequence and bar code, and as a result small variations in probe efficiency can result in count variation between probes even when targeting genomic regions of equal copy number. However, this difference in counting efficiency will be constant for a given probe over all samples analyzed. Therefore, highly accurate copy number estimates can be generated by simply taking the ratio of counts from test samples to the counts of a fixed reference sample(s), and calculating copy numbers relative to that reference sample.

The example analysis that follows will assume a copy number of 2 for all genomic loci being assayed in the reference sample. In a later section (**Reference Sample Adjustments on page 10**), we will consider alternative analysis strategies for genomic loci that differ from 2 copies in the reference sample(s).

**NOTE:** If the copy number of your reference sample is not known, the analysis method presented here will only estimate the copy number relative to the unknown reference; an absolute integer copy number prediction will not be possible.

First, start a new sheet for copy number calculations and select your reference sample. In the example below, the reference sample will be Sample 1 (Column D). Next, divide each test samples probe value (INV-normalized counts) by the corresponding probes in the reference samples' INV-normalized counts. For autosomal chromosomes (1-22), multiply this quotient by 2 to account for the presence of two chromosomal copies in the diploid reference sample. (**Hint:** Use the "$" shortcut command to hold the reference sample column constant in the formula.)

**FIGURE 1.9**

| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 21 | **Reporter Counts** | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 22 | *Code Class* | *Name* | *Accession* | | | | | | |
| 23 | Invariant | _INVCONTROL-1\|+\|Hg18\|chr1:97239055-97239154 | _INVCONTROL | ='INV norm example'!$D23/'INV norm example'!D23*2 | | | | | 2.1 |
| 24 | Invariant | _INVCONTROL-10\|+\|Hg18\|chr10:76411622-76411718 | _INVCONTROL | 2.0 | 2.4 | 1.8 | 1.9 | 1.9 | 1.9 |
| 25 | Invariant | _INVCONTROL-16\|+\|Hg18\|chr16:11026537-11026636 | _INVCONTROL | 2.0 | 1.7 | 1.8 | 1.7 | 1.6 | 1.6 |
| 26 | Invariant | _INVCONTROL-17\|+\|Hg18\|chr17:60617472-60617571 | _INVCONTROL | 2.0 | 2.1 | 2.2 | 2.0 | 2.1 | 2.1 |
| 27 | Invariant | _INVCONTROL-19\|+\|Hg18\|chr19:38818850-38818949 | _INVCONTROL | 2.0 | 2.0 | 1.8 | 1.9 | 2.0 | 2.1 |
| 28 | Invariant | _INVCONTROL-2\|+\|Hg18\|chr2:137221998-137222092 | _INVCONTROL | 2.0 | 2.0 | 1.9 | 2.0 | 1.8 | 2.0 |
| 29 | Invariant | _INVCONTROL-20\|+\|Hg18\|chr20:10504898-10504992 | _INVCONTROL | 2.0 | 2.1 | 1.9 | 1.8 | 1.9 | 2.0 |
| 30 | Invariant | _INVCONTROL-22\|+\|Hg18\|chr22:42509372-42509471 | _INVCONTROL | 2.0 | 2.0 | 2.2 | 2.1 | 2.0 | 2.1 |
| 31 | Invariant | _INVCONTROL-5\|+\|Hg18\|chr5:71261236-71261335 | _INVCONTROL | 2.0 | 1.8 | 2.1 | 2.1 | 2.1 | 2.0 |
| 32 | Invariant | _INVCONTROL-7\|+\|Hg18\|chr7:41541153-41541252 | _INVCONTROL | 2.0 | 1.9 | 1.9 | 2.0 | 1.9 | 2.0 |

## X and Y Chromosome Copy Number Calculation

The formula for determining copy number of the X and Y chromosomes must be adjusted depending on the gender of the reference sample. If the reference sample is female, the formulas should reflect two copies of the X chromosome and 0 copies of the Y chromosome as shown on **Figure 1.10**.

**FIGURE 1.10**

| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 21 | **Reporter Counts** | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 22 | *Code Class* | *Name* | *Accession* | | | | | | |
| 23 | Invariant | _INVCONTROL-1\|+\|Hg18\|chr1:97239055-97239154 | _INVCONTROL | 2.0 | 2.2 | 2.2 | 2.2 | 2.4 | 2.1 |
| 24 | Invariant | _INVCONTROL-10\|+\|Hg18\|chr10:76411622-76411718 | _INVCONTROL | 2.0 | 2.4 | 1.8 | 1.9 | 1.9 | 1.9 |
| 25 | Invariant | _INVCONTROL-16\|+\|Hg18\|chr16:11026537-11026636 | _INVCONTROL | 2.0 | 1.7 | 1.8 | 1.7 | 1.6 | 1.6 |
| 26 | Invariant | _INVCONTROL-17\|+\|Hg18\|chr17:60617472-60617571 | _INVCONTROL | 2.0 | 2.1 | 2.2 | 2.0 | 2.1 | 2.1 |
| 27 | Invariant | _INVCONTROL-19\|+\|Hg18\|chr19:38818850-38818949 | _INVCONTROL | 2.0 | 2.0 | 1.8 | 1.9 | 2.0 | 2.1 |
| 28 | Invariant | _INVCONTROL-2\|+\|Hg18\|chr2:137221998-137222092 | _INVCONTROL | 2.0 | 2.0 | 1.9 | 2.0 | 1.8 | 2.0 |
| 29 | Invariant | _INVCONTROL-20\|+\|Hg18\|chr20:10504898-10504992 | _INVCONTROL | 2.0 | 2.1 | 1.9 | 1.8 | 1.9 | 2.0 |
| 30 | Invariant | _INVCONTROL-22\|+\|Hg18\|chr22:42509372-42509471 | _INVCONTROL | 2.0 | 2.0 | 2.2 | 2.1 | 2.0 | 2.1 |
| 31 | Invariant | _INVCONTROL-5\|+\|Hg18\|chr5:71261236-71261335 | _INVCONTROL | 2.0 | 1.8 | 2.1 | 2.1 | 2.1 | 2.0 |
| 32 | Invariant | _INVCONTROL-7\|+\|Hg18\|chr7:41541153-41541252 | _INVCONTROL | 2.0 | 1.9 | 1.9 | 2.0 | 1.9 | 2.0 |
| 33 | Endogenous | probe1\|+\|Hg18\|chr1: *coordinates* | CNV region 1 | 0.6 | 3.2 | 2.2 | 2.6 | 3.0 | 3.9 |
| 34 | Endogenous | probe2\|+\|Hg18\|chr8: *coordinates* | CNV region 2 | 2.0 | 3.1 | 0.7 | 1.0 | 1.0 | 1.4 |
| 35 | Endogenous | probe3\|+\|Hg18\|chr10: *coordinates* | CNV region 3 | 2.0 | 3.0 | 1.4 | 1.9 | 2.1 | 2.7 |
| 36 | Endogenous | probe4\|+\|Hg18\|chr12: *coordinates* | CNV region 4 | 2.0 | 3.2 | 1.0 | 1.3 | 1.3 | 1.8 |
| 37 | Endogenous | probe5\|+\|Hg18\|chrX:111441962-111442061 | CNV region 5 | ='INV norm example'!D37/'INV norm example'!$D37*2 | | | | | 2.1 |
| 38 | Endogenous | probe6\|+\|Hg18\|chrY:2715200-2715287 | CNV region 6 | 0.0 | 1.0 | 1.0 | 1.1 | 0.0 | 0.0 |

In order to generate meaningful copy number estimates for the Y chromosome probe, it is best to use a reference sample that contains a Y chromosome (male). The formula for Y chromosome probes can then be adjusted to use this male reference to calculate copy numbers for only the Y chromosome probe(s). In the example below, the reference for the Y chromosome probe has been switched to Sample 2 (Column E):

**FIGURE 1.11**

| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 21 | **Reporter Counts** | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 22 | *Code Class* | *Name* | *Accession* | | | | | | |
| 23 | Invariant | _INVCONTROL-1\|+\|Hg18\|chr1:97239055-97239154 | _INVCONTROL | 2.0 | 2.2 | 2.2 | 2.2 | 2.4 | 2.1 |
| 24 | Invariant | _INVCONTROL-10\|+\|Hg18\|chr10:76411622-76411718 | _INVCONTROL | 2.0 | 2.4 | 1.8 | 1.9 | 1.9 | 1.9 |
| 25 | Invariant | _INVCONTROL-16\|+\|Hg18\|chr16:11026537-11026636 | _INVCONTROL | 2.0 | 1.7 | 1.8 | 1.7 | 1.6 | 1.6 |
| 26 | Invariant | _INVCONTROL-17\|+\|Hg18\|chr17:60617472-60617571 | _INVCONTROL | 2.0 | 2.1 | 2.2 | 2.0 | 2.1 | 2.1 |
| 27 | Invariant | _INVCONTROL-19\|+\|Hg18\|chr19:38818850-38818949 | _INVCONTROL | 2.0 | 2.0 | 1.8 | 1.9 | 2.0 | 2.1 |
| 28 | Invariant | _INVCONTROL-2\|+\|Hg18\|chr2:137221998-137222092 | _INVCONTROL | 2.0 | 2.0 | 1.9 | 2.0 | 1.8 | 2.0 |
| 29 | Invariant | _INVCONTROL-20\|+\|Hg18\|chr20:10504898-10504992 | _INVCONTROL | 2.0 | 2.1 | 1.9 | 1.8 | 1.9 | 2.0 |
| 30 | Invariant | _INVCONTROL-22\|+\|Hg18\|chr22:42509372-42509471 | _INVCONTROL | 2.0 | 2.0 | 2.2 | 2.1 | 2.0 | 2.1 |
| 31 | Invariant | _INVCONTROL-5\|+\|Hg18\|chr5:71261236-71261335 | _INVCONTROL | 2.0 | 1.8 | 2.1 | 2.1 | 2.1 | 2.0 |
| 32 | Invariant | _INVCONTROL-7\|+\|Hg18\|chr7:41541153-41541252 | _INVCONTROL | 2.0 | 1.9 | 1.9 | 2.0 | 1.9 | 2.0 |
| 33 | Endogenous | probe1\|+\|Hg18\|chr1: *coordinates* | CNV region 1 | 0.6 | 3.2 | 2.2 | 2.6 | 3.0 | 3.9 |
| 34 | Endogenous | probe2\|+\|Hg18\|chr8: *coordinates* | CNV region 2 | 2.0 | 3.1 | 0.7 | 1.0 | 1.0 | 1.4 |
| 35 | Endogenous | probe3\|+\|Hg18\|chr10: *coordinates* | CNV region 3 | 2.0 | 3.0 | 1.4 | 1.9 | 2.1 | 2.7 |
| 36 | Endogenous | probe4\|+\|Hg18\|chr12: *coordinates* | CNV region 4 | 2.0 | 3.2 | 1.0 | 1.3 | 1.3 | 1.8 |
| 37 | Endogenous | probe5\|+\|Hg18\|chrX:111441962-111442061 | CNV region 5 | 2.0 | 1.2 | 1.4 | 0.9 | 2.1 | 2.1 |
| 38 | Endogenous | probe6\|+\|Hg18\|chrY:2715200-2715287 | CNV re | ='INV norm example'!E38/'INV | norm example'!$E38 | | | 0.0 | 0.0 |

## Averaging Copy Number Estimates by Genomic Region

If your nCounter Custom CNV CodeSet contains multiple probes for a single genomic locus, it may be desirable to generate an average copy number estimate value based on all probes for that particular locus. This average value can then be used to generate an integer copy number assignment. If you are using a single probe per region, proceed to **Generating Integer Copy Number Calls**, below, for instructions on how to round the estimated copy number.

To average probes for each locus, create a new spreadsheet. For each genomic region (locus), calculate the average of copy number estimate values using the "=AVERAGE" function as described in **Normalization to Invariant Probes on page 5**.

## Generating Integer Copy Number Calls

To convert copy number estimates to integer copy number predictions, the copy number estimate values must be rounded. The simplest method is to round each estimated value to the nearest integer, using an "IF" function in Excel. In the following example we demonstrate an integer copy number +/- 0.4, although each investigator should determine the appropriate rounding criteria based on their own specific data analysis requirements.

**NOTE:** Copy number estimates that are half-integer values (e.g., 1.5, 2.5, 3.5) will require further interpretation by the investigator to determine the integer copy number value. In the following example, such values are returned as the decimal copy number estimate value and are not rounded to the nearest integer.



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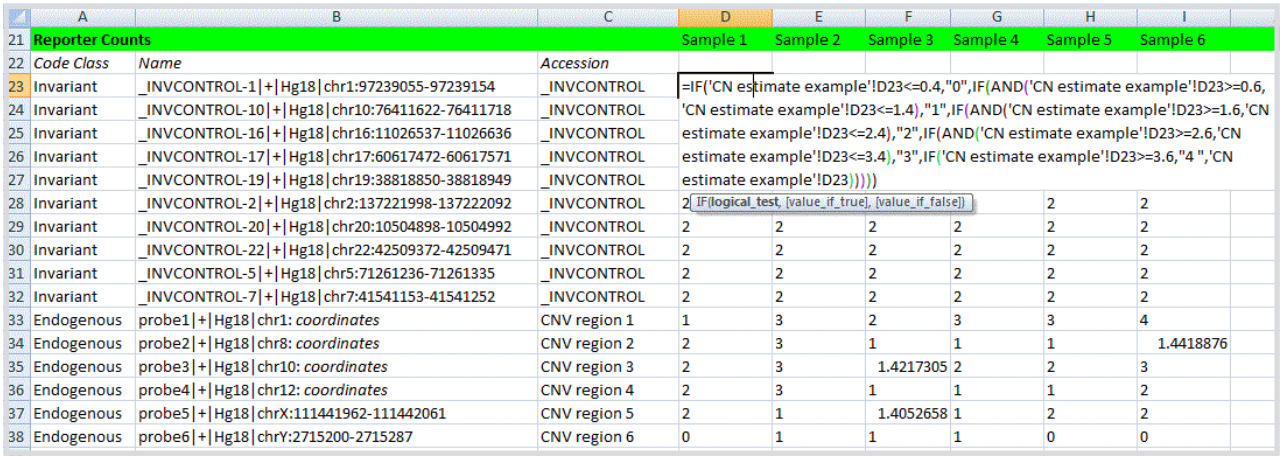
On a new sheet, enter an "IF" formula in the cell corresponding to the first probe and first sample following the example shown below. The values entered in the formula can be adjusted to alter the rounding criteria. For example, values between 0.8 and 1.2 (rather than 0.6 and 1.4) can be rounded to 1. Once the formula has been entered, click on the lower-right corner of the cell and drag the formula down (to apply to all Invariant and Endogenous probes), then across to apply to all samples.

**FIGURE 1.12**



| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 21 | **Reporter Counts** | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 22 | *Code Class* | *Name* | *Accession* | | | | | | |
| 23 | Invariant | _INVCONTROL-1\|+\|Hg18\|chr1:97239055-97239154 | _INVCONTROL | =IF('CN estimate example'!D23<=0.4,"0",IF(AND('CN estimate example'!D23>=0.6, | | | | | |
| 24 | Invariant | _INVCONTROL-10\|+\|Hg18\|chr10:76411622-76411718 | _INVCONTROL | 'CN estimate example'!D23<=1.4),"1",IF(AND('CN estimate example'!D23>=1.6,'CN | | | | | |
| 25 | Invariant | _INVCONTROL-16\|+\|Hg18\|chr16:11026537-11026636 | _INVCONTROL | estimate example'!D23<=2.4),"2",IF(AND('CN estimate example'!D23>=2.6,'CN | | | | | |
| 26 | Invariant | _INVCONTROL-17\|+\|Hg18\|chr17:60617472-60617571 | _INVCONTROL | estimate example'!D23<=3.4),"3",IF('CN estimate example'!D23>=3.6,"4 ','CN | | | | | |
| 27 | Invariant | _INVCONTROL-19\|+\|Hg18\|chr19:38818850-38818949 | _INVCONTROL | estimate example'!D23))))) | | | | | |
| 28 | Invariant | _INVCONTROL-2\|+\|Hg18\|chr2:137221998-137222092 | _INVCONTROL | 2 IF(logical_test, [value_if_true], [value_if_false]) | | | | 2 | 2 |
| 29 | Invariant | _INVCONTROL-20\|+\|Hg18\|chr20:10504898-10504992 | _INVCONTROL | 2 | 2 | 2 | 2 | 2 | 2 |
| 30 | Invariant | _INVCONTROL-22\|+\|Hg18\|chr22:42509372-42509471 | _INVCONTROL | 2 | 2 | 2 | 2 | 2 | 2 |
| 31 | Invariant | _INVCONTROL-5\|+\|Hg18\|chr5:71261236-71261335 | _INVCONTROL | 2 | 2 | 2 | 2 | 2 | 2 |
| 32 | Invariant | _INVCONTROL-7\|+\|Hg18\|chr7:41541153-41541252 | _INVCONTROL | 2 | 2 | 2 | 2 | 2 | 2 |
| 33 | Endogenous | probe1\|+\|Hg18\|chr1: *coordinates* | CNV region 1 | 1 | 3 | 2 | 3 | 3 | 4 |
| 34 | Endogenous | probe2\|+\|Hg18\|chr8: *coordinates* | CNV region 2 | 2 | 3 | 1 | 1 | 1 | 1.4418876 |
| 35 | Endogenous | probe3\|+\|Hg18\|chr10: *coordinates* | CNV region 3 | 2 | 3 | 1.4217305 | 2 | 2 | 3 |
| 36 | Endogenous | probe4\|+\|Hg18\|chr12: *coordinates* | CNV region 4 | 2 | 3 | 1 | 1 | 1 | 2 |
| 37 | Endogenous | probe5\|+\|Hg18\|chrX:111441962-111442061 | CNV region 5 | 2 | 1 | 1.4052658 | 1 | 2 | 2 |
| 38 | Endogenous | probe6\|+\|Hg18\|chrY:2715200-2715287 | CNV region 6 | 0 | 1 | 1 | 1 | 0 | 0 |

Here we have presented a simple three-step method for generating integer copy number calls from the nCounter CNV Assay raw data: normalization to INV probe counts, copy number estimation relative to a reference sample, and conversion to integer copy number calls.

In the next section (**Reference Sample Adjustments**) we will consider situations when the copy number of the reference sample differs from 2, and when it is desirable to use multiple reference samples.

## Reference Sample Adjustments

In some cases, the reference sample selected may contain genomic regions with a copy number of 0 (deletion), 1 (single-copy), or greater than 2 copies. Since it is not possible to calculate copy number estimates using a reference sample with 0 copies at a particular locus, it is necessary to change the reference to a sample that has counts registered for that specific region, and that has a known copy number.

### Adjusting the Copy Number Estimate Calculation

After the appropriate reference sample has been identified, it may be necessary to adjust the formula for calculating the copy number estimate. If the reference sample copy number is 1 for a particular genomic region, the formula for the corresponding probes can be altered by deleting the multiplication factor of "2". In the example below, the reference sample for probe1 (Chr1) is changed to Sample2 (Column E) and the copy number formula is adjusted for a copy number of "1":

**NOTE:** It may not be necessary to change the reference sample, only to alter the known copy number of the original reference sample following the same method outlined here.

FIGURE 1.13

| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 21 | **Reporter Counts** | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 22 | *Code Class* | *Name* | *Accession* | | | | | | |
| 23 | Invariant | _INVCONTROL-1\|+\|Hg18\|chr1:97239055-97239154 | _INVCONTROL | 2.0 | 2.2 | 2.2 | 2.2 | 2.4 | 2.1 |
| 24 | Invariant | _INVCONTROL-10\|+\|Hg18\|chr10:76411622-76411718 | _INVCONTROL | 2.0 | 2.4 | 1.8 | 1.9 | 1.9 | 1.9 |
| 25 | Invariant | _INVCONTROL-16\|+\|Hg18\|chr16:11026537-11026636 | _INVCONTROL | 2.0 | 1.7 | 1.8 | 1.7 | 1.6 | 1.6 |
| 26 | Invariant | _INVCONTROL-17\|+\|Hg18\|chr17:60617472-60617571 | _INVCONTROL | 2.0 | 2.1 | 2.2 | 2.0 | 2.1 | 2.1 |
| 27 | Invariant | _INVCONTROL-19\|+\|Hg18\|chr19:38818850-38818949 | _INVCONTROL | 2.0 | 2.0 | 1.8 | 1.9 | 2.0 | 2.1 |
| 28 | Invariant | _INVCONTROL-2\|+\|Hg18\|chr2:137221998-137222092 | _INVCONTROL | 2.0 | 2.0 | 1.9 | 2.0 | 1.8 | 2.0 |
| 29 | Invariant | _INVCONTROL-20\|+\|Hg18\|chr20:10504898-10504992 | _INVCONTROL | 2.0 | 2.1 | 1.9 | 1.8 | 1.9 | 2.0 |
| 30 | Invariant | _INVCONTROL-22\|+\|Hg18\|chr22:42509372-42509471 | _INVCONTROL | 2.0 | 2.0 | 2.2 | 2.1 | 2.0 | 2.1 |
| 31 | Invariant | _INVCONTROL-5\|+\|Hg18\|chr5:71261236-71261335 | _INVCONTROL | 2.0 | 1.8 | 2.1 | 2.1 | 2.1 | 2.0 |
| 32 | Invariant | _INVCONTROL-7\|+\|Hg18\|chr7:41541153-41541252 | _INVCONTROL | 2.0 | 1.9 | 1.9 | 2.0 | 1.9 | 2.0 |
| 33 | Endogenous | probe1\|+\|Hg18\|chr1: *coordinates* | CNV region 1 | ='INV norm | example'!D33/'INV norm example'!$E33 | | | | 3.9 |
| 34 | Endogenous | probe2\|+\|Hg18\|chr8: *coordinates* | CNV region 2 | 2.0 | 3.1 | 0.7 | 1.0 | 1.0 | 1.4 |
| 35 | Endogenous | probe3\|+\|Hg18\|chr10: *coordinates* | CNV region 3 | 2.0 | 3.0 | 1.4 | 1.9 | 2.1 | 2.7 |
| 36 | Endogenous | probe4\|+\|Hg18\|chr12: *coordinates* | CNV region 4 | 2.0 | 3.2 | 1.0 | 1.3 | 1.3 | 1.8 |
| 37 | Endogenous | probe5\|+\|Hg18\|chrX:111441962-111442061 | CNV region 5 | 2.0 | 1.2 | 1.4 | 0.9 | 2.1 | 2.1 |
| 38 | Endogenous | probe6\|+\|Hg18\|chrY:2715200-2715287 | CNV region 6 | 0.0 | 1.0 | 1.0 | 1.1 | 0.0 | 0.0 |

If the copy number of the new reference is 3, the formula can be adjusted by changing the multiplication factor to "3" to generate the correct copy number. In the following example, the reference sample is Sample 1, but the formula is adjusted by adding a multiplication factor of 3 to reflect 3 copies of probe region1 in the reference sample:

**FIGURE 1.14**

| | A | B | C | D | E | F | G | |
|---|---|---|---|---|---|---|---|---|
| 21 | **Reporter Counts** | | | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Samp |
| 22 | *Code Class* | *Name* | *Accession* | | | | | |
| 23 | Invariant | _INVCONTROL-1\|+\|Hg18\|chr1:97239055-97239154 | _INVCONTROL | 2.0 | 2.2 | 2.2 | 2.2 | |
| 24 | Invariant | _INVCONTROL-10\|+\|Hg18\|chr10:76411622-76411718 | _INVCONTROL | 2.0 | 2.4 | 1.8 | 1.9 | |
| 25 | Invariant | _INVCONTROL-16\|+\|Hg18\|chr16:11026537-11026636 | _INVCONTROL | 2.0 | 1.7 | 1.8 | 1.7 | |
| 26 | Invariant | _INVCONTROL-17\|+\|Hg18\|chr17:60617472-60617571 | _INVCONTROL | 2.0 | 2.1 | 2.2 | 2.0 | |
| 27 | Invariant | _INVCONTROL-19\|+\|Hg18\|chr19:38818850-38818949 | _INVCONTROL | 2.0 | 2.0 | 1.8 | 1.9 | |
| 28 | Invariant | _INVCONTROL-2\|+\|Hg18\|chr2:137221998-137222092 | _INVCONTROL | 2.0 | 2.0 | 1.9 | 2.0 | |
| 29 | Invariant | _INVCONTROL-20\|+\|Hg18\|chr20:10504898-10504992 | _INVCONTROL | 2.0 | 2.1 | 1.9 | 1.8 | |
| 30 | Invariant | _INVCONTROL-22\|+\|Hg18\|chr22:42509372-42509471 | _INVCONTROL | 2.0 | 2.0 | 2.2 | 2.1 | |
| 31 | Invariant | _INVCONTROL-5\|+\|Hg18\|chr5:71261236-71261335 | _INVCONTROL | 2.0 | 1.8 | 2.1 | 2.1 | |
| 32 | Invariant | _INVCONTROL-7\|+\|Hg18\|chr7:41541153-41541252 | _INVCONTROL | 2.0 | 1.9 | 1.9 | 2.0 | |
| 33 | Endogenous | probe1\|+\|Hg18\|chr1: *coordinates* | CNV region 1 | ='INV norm example'!D33/'INV norm example'!$D33*3 | | | | |
| 34 | Endogenous | probe2\|+\|Hg18\|chr8: *coordinates* | CNV region 2 | 2.0 | 3.1 | 0.7 | 1.0 | |
| 35 | Endogenous | probe3\|+\|Hg18\|chr10: *coordinates* | CNV region 3 | 2.0 | 3.0 | 1.4 | 1.9 | |
| 36 | Endogenous | probe4\|+\|Hg18\|chr12: *coordinates* | CNV region 4 | 2.0 | 3.2 | 1.0 | 1.3 | |
| 37 | Endogenous | probe5\|+\|Hg18\|chrX:111441962-111442061 | CNV region 5 | 2.0 | 1.2 | 1.4 | 0.9 | |
| 38 | Endogenous | probe6\|+\|Hg18\|chrY:2715200-2715287 | CNV region 6 | 0.0 | 1.0 | 1.0 | 1.1 | |

The adjusted formula can then be copied to the appropriate cells for each probe by clicking on the lower right corner of the highlighted cell and dragging over the cells you would like to change.

## Reference Documents and Support

For additional information on the nCounter CNV Assay and data analysis, please refer to these documents available for download at **www.nanostring.com**:

- **nCounter® CNV Collector Tool User Manual**
- **nCounter® Custom CNV Assay Manual**

### Contacting Support

For questions about the nCounter® CNV Assay, nCounter® CNV Collector Tool or data analysis, please contact:

**support@nanostring.com**

**Phone:** 206-378-NANO

or contact your NanoString Field Applications Scientist.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