Evaluation of Infinium Genotyping Assay Controls Training Guide

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INTRODUCTION

This Guide and the Online Course

This Training Guide is a companion to the online course Evaluation of Infinium Genotyping Assay Controls.

It contains the same information presented in the online modules, in a printable format. This document is designed to be printed double-sided and in color.

Accessing the Online Course

You can access the online modules of this course from the Illumina website.

To launch a training module:

- Navigate to: http://support.illumina.com/training/online-courses/array.html
- Click the module title.

Evaluation of Infinium Genotyping Assay Controls

Module Objectives

By the end of this module, you will be able to:

- Describe the purpose of the Infinium Genotyping Assay controls.
- List sample-dependent and sample-independent Infinium controls.
- Describe how to view the Infinium controls in GenomeStudio.
- Describe expected outcomes for each of the controls.

Evaluation of Infinium Genotyping Assay Controls Module Overview

About this Module

This module provides a guide on how to view controls used in the Infinium Genotyping assay and their expected outcomes. It explains the different types of controls and identifies the assay workflow steps where they come into play.

About the Training Data Set

The training data set shown in this module has been generating using HumanOmni5-Quad BeadChips. Note that call rates, absolute intensities and background thresholds reported in any Infinium project may be different from the data shown in the examples in this module.

You can access the HumanOmni5-Quad Demo Workspace at http://support.illumina.com/array/array_kits/humanomni5-quad_beadchip_kit/downloads.html.

Infinium Controls

About the Infinium Controls

Infinium microarrays provide tools to genotype with unparalleled accuracy and reproducibility. All Infinium BeadChips are equipped with a set of internal control probes designed to support quality control of the assay's stringent performance criteria and to demonstrate its robustness.

High quality samples generally yield unambiguous genotype calls with average call rates of >99%. Built-in Infinium controls help to identify samples for which data characteristics are significantly different, and may need to be excluded as outliers from further analysis.

Also, Infinium controls facilitate troubleshooting by linking possible root causes to specific steps in the assay protocol.

As intensity levels may be different for any given Infinium project, Infinium controls are not designed to perform quality control based on specific thresholds. Instead, Infinium controls are evaluated based on relative intensities.

Infinium Protocol and Controls

Figure 1 summarizes the key steps of the Infinium Genotyping assay and demonstrates where internal controls come into play. While individual controls can be particularly informative of certain steps in the assay workflow, they may still be affected by performance of other steps in the workflow. Therefore, built-in Infinium controls are most useful when assessed in combination with each other.

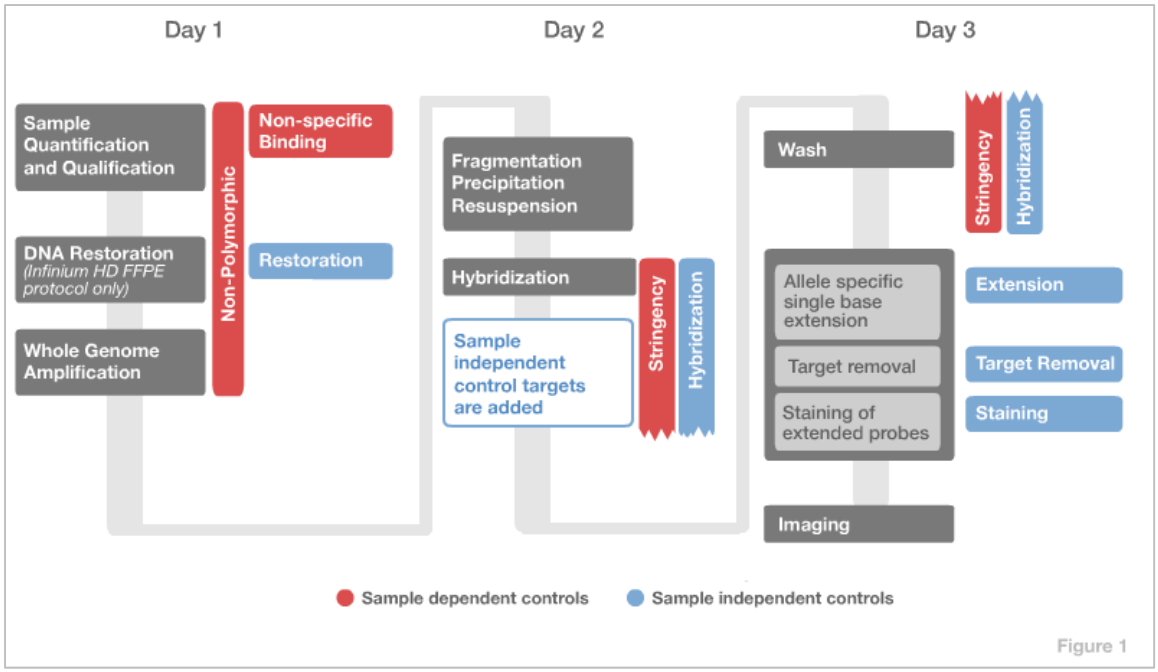


Figure 1: Overview of Infinium Genotyping Assay Protocol and Steps Where Built-In Controls Come Into Play

Sample-Dependent and Sample-Independent Controls

The Infinium assay controls are comprised of **sample-independent controls** (shown in blue in Figure 1) and **sample-dependent controls** (shown in red in Figure 1).

Sample-independent controls evaluate BeadChip and reagent performance, efficiency of hybridization, and the staining process. They include Staining Controls, Extension Controls, Target Removal Controls, Hybridization Controls, and the Restoration Control. The Restoration Control is only informative if following the Infinium Formalin-Fixed Paraffin-Embedded (FFPE) workflow.

The sample-dependent controls are used to evaluate sample quality and performance. They include Stringency Controls, Non-Specific Binding Controls, and Non-Polymorphic Controls. Sample-dependent Stringency and Non-Polymorphic Control probes specifically target human DNA and are not informative when working with non-human BeadChip products.

See Appendix A for detailed information on each Infinium control bead type and its function.

Viewing the Controls in GenomeStudio

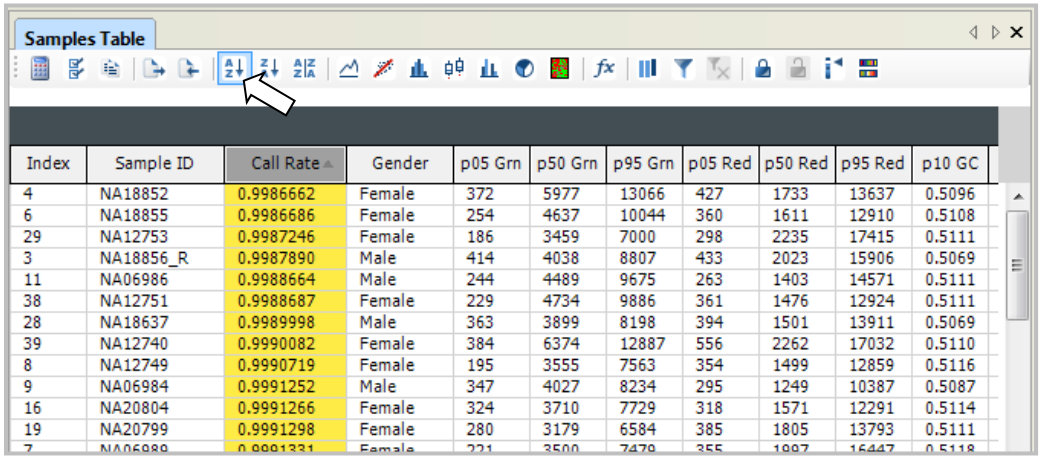
In this module, we include data generated with HumanOmni5-Quad BeadChips, loaded with HapMap samples.

Sorting by Call Rate

First, we sort the samples by call rate so that samples with the lowest call rates display on the left side of each control panel. It is normal to observe some variation between signal intensity of data points in the controls. This signal noise in the Infinium assay is accounted for by data normalization and may not necessarily impact performance. Plotting the Controls Dashboard in this way allows one to distinguish common variations from true data outliers, and to correlate changes in the appearance of controls to potential impact on assay performance and sample call rates.

To sort the samples by call rate:

- In the Samples Table, select the Call Rate column.
- Press the Sort Ascending (A - Z) button.



Index	Sample ID	Call Rate	Gender	p05 Grn	p50 Grn	p95 Grn	p05 Red	p50 Red	p95 Red	p10 GC
4	NA18852	0.9986662	Female	372	5977	13066	427	1733	13637	0.5096
6	NA18855	0.9986686	Female	254	4637	10044	360	1611	12910	0.5108
29	NA12753	0.9987246	Female	186	3459	7000	298	2235	17415	0.5111
3	NA18856_R	0.9987890	Male	414	4038	8807	433	2023	15906	0.5069
11	NA06986	0.9988664	Male	244	4489	9675	263	1403	14571	0.5111
38	NA12751	0.9988687	Female	229	4734	9886	361	1476	12924	0.5111
28	NA18637	0.9989998	Male	363	3899	8198	394	1501	13911	0.5069
39	NA12740	0.9990082	Female	384	6374	12887	556	2262	17032	0.5110
8	NA12749	0.9990719	Female	195	3555	7563	354	1499	12859	0.5116
9	NA06984	0.9991252	Male	347	4027	8234	295	1249	10387	0.5087
16	NA20804	0.9991266	Female	324	3710	7729	318	1571	12291	0.5114
19	NA20799	0.9991298	Female	280	3179	6584	385	1805	13793	0.5111
7	NA06989	0.9991331	Female	221	3500	7479	355	1997	16447	0.5118

Figure 2: Sorting Samples by Call Rate

Similarly, samples can also be sorted by BeadChip barcode, Sentrix position, sample plate ID, sample well, etc., to determine possible correlations and patterns in the appearance of controls.

Opening the Controls Dashboard

To open the Controls Dashboard:

- Select Analysis > View Controls Dashboard. (See Figure 3.)
- The Controls Dashboard displays. Scroll to view all of the controls.
- Infinium controls data can be exported in table format by clicking File and Export data in the Controls Dashboard Window. (See Figure 4.)

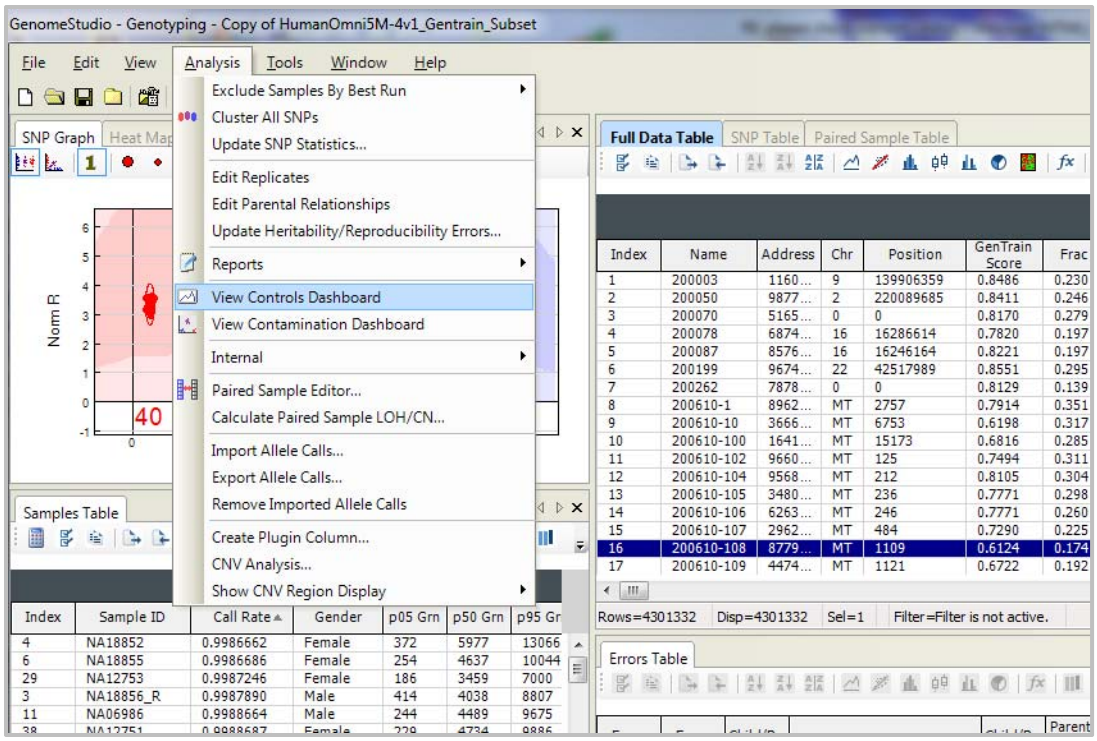


Figure 3: Opening the Controls Dashboard in GenomeStudio

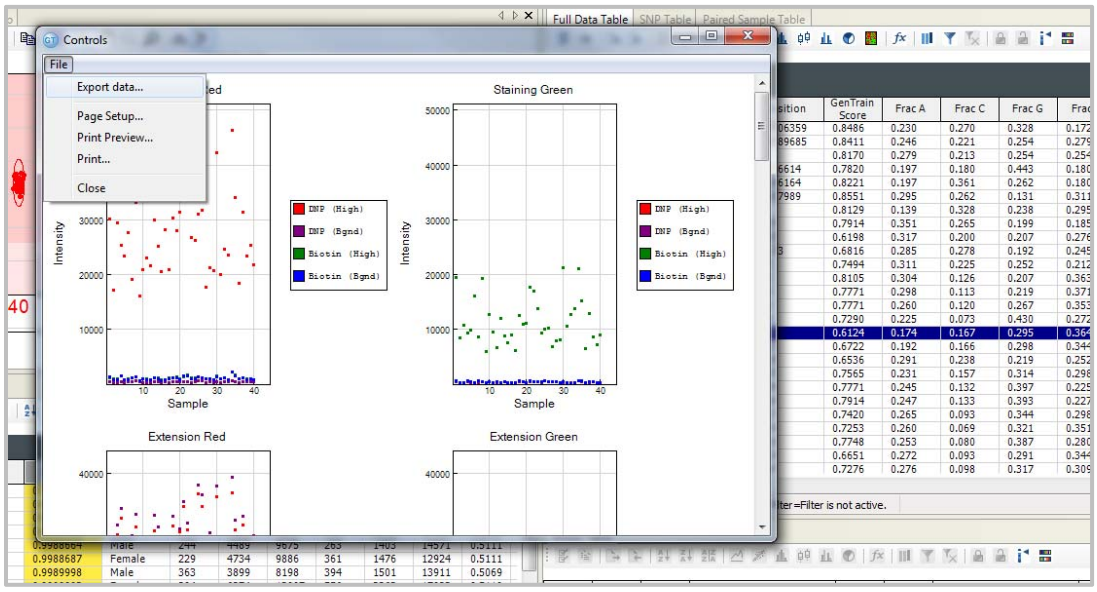


Figure 4: Exporting Controls Data

Evaluating Controls

Evaluation Overview

Figures 6 through 13 present screenshots of the Controls Dashboard for the training data set. They illustrate the expected outcome for each type of Infinium control.

Signal Intensity in Infinium Assays

Note that the absolute intensities and background thresholds reported in the Controls Dashboard of any Infinium project may be different from the data shown in this example.

Signal intensity in the Infinium assay is subject to variations that are rooted in, but not limited to:

- Variations in DNA preparation methods, sources, or tissue types
- Variability in which individual users perform the assay
- Normal lot-to-lot variations and variations between scanners that do not impact results

Accordingly, sample performance should not be assessed based on absolute intensities of controls in an individual batch. Instead, for effective evaluation of the Controls Dashboard, it is crucial to assess **relative intensities** of signal to background.

As a quality control best practice, trends in intensity changes across batches should be monitored.

Relative Intensities - Example

Here we show how to assess relative intensities of signal to background in a given panel.

The color bands on the right in Figure 5, illustrate what should be considered high signal intensity, low signal intensity, and background for the Staining Red Control panel.

Please keep in mind that absolute intensity levels for high, low, and background intensities for this and other panels may differ for your project.

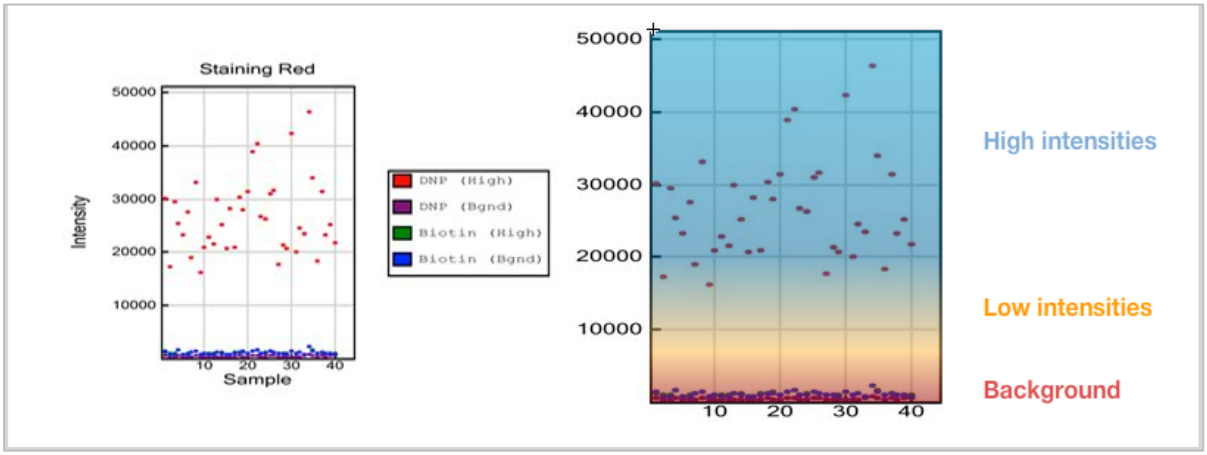


Figure 5: Relative Intensities for an Infinium Control

Expected Control Outcomes

In this section, we'll discuss the expected outcome for each of the controls. Some controls are monitored in only one color channel. Several key steps in the Infinium assay require evaluation of both the red and green color channels. For these instances, both red and green channel controls are included.

Staining Controls (Sample-Independent)

The **Staining Controls** assess the efficiency of the staining process during the X-Stain protocol.

See Appendix A for detailed information for this control.

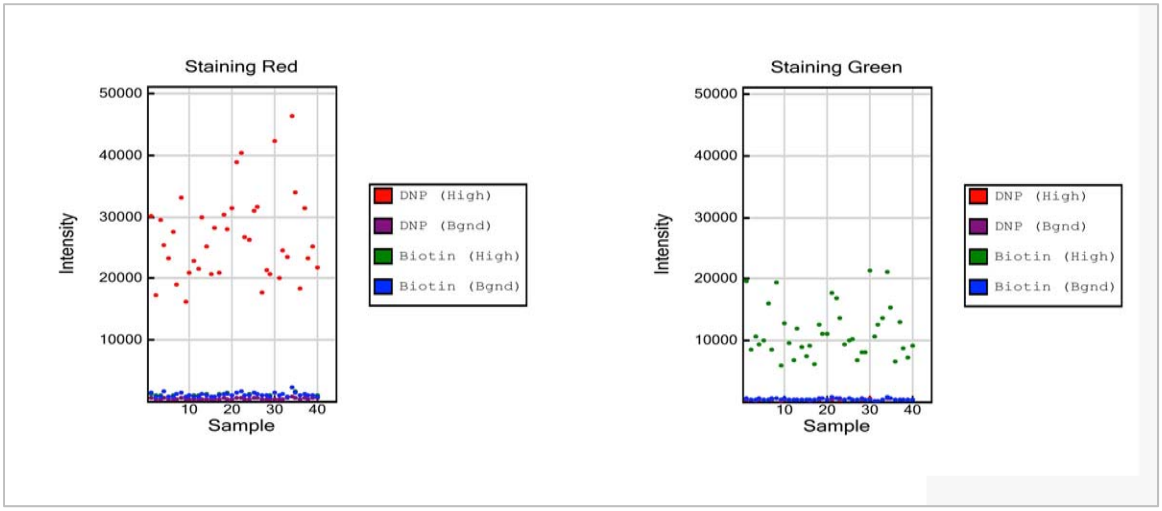


Figure 6: Staining Controls

Controls	Expected Outcome
Staining Red	Strong positive signals are expected for the dinitrophenyl (DNP) (High) data points, while background is expected for the DNP (Bgnd), Biotin (High), and Biotin (Bgnd) signals.
Staining Green	Strong positive signals are expected for the Biotin (High) data points, while background is expected for the DNP (High), DNP (Bgnd), and Biotin (Bgnd) data points.

No comparison is made between the levels of positive signal between the red and green channels, nor is there a specific threshold value for the intensities.

Extension Controls (Sample-Independent)

The **Extension Controls** test the efficiency of single base extension during the X-stain protocol.

See Appendix A for detailed information for this control.

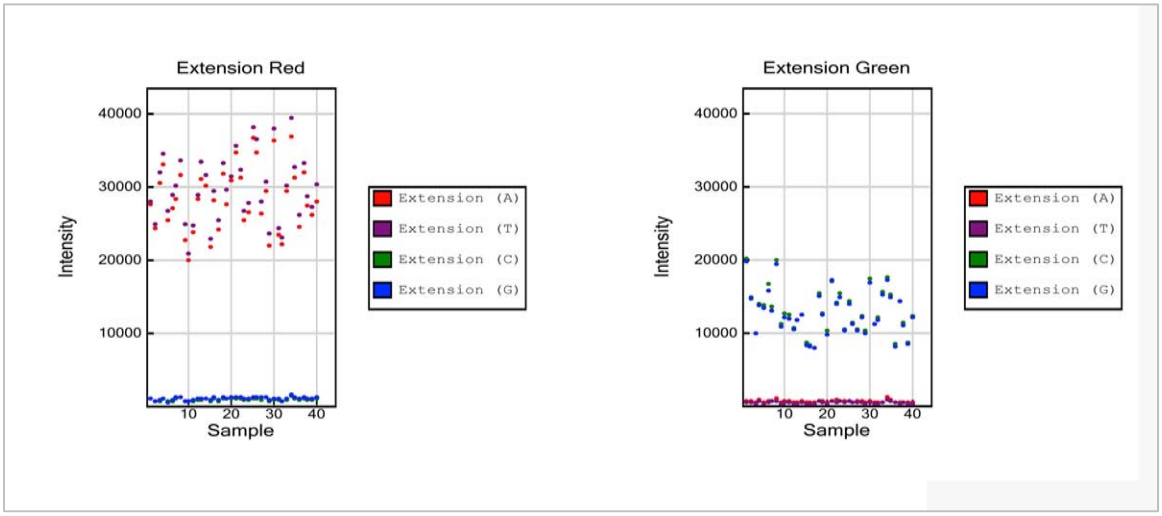


Figure 7: Extension Controls

Controls	Expected Outcome
Extension Red	Strong positive signals are expected for Extension (A) and Extension (T) data points, while signals for Extension (C) and Extension (G) are expected to be at background levels.
Extension Green	Strong positive signals are expected for Extension (C) and Extension (G) data points, while signals for Extension (A) and Extension (T) are expected to be at background levels.

No comparison is made between the levels of positive signal between the red and green channels, nor is there a specific threshold value for the intensities.

Target Removal Controls (Sample-Independent)

The **Target Removal Controls** test the efficiency of stripping off DNA templates after the extension reaction.

See Appendix A for detailed information for this control.

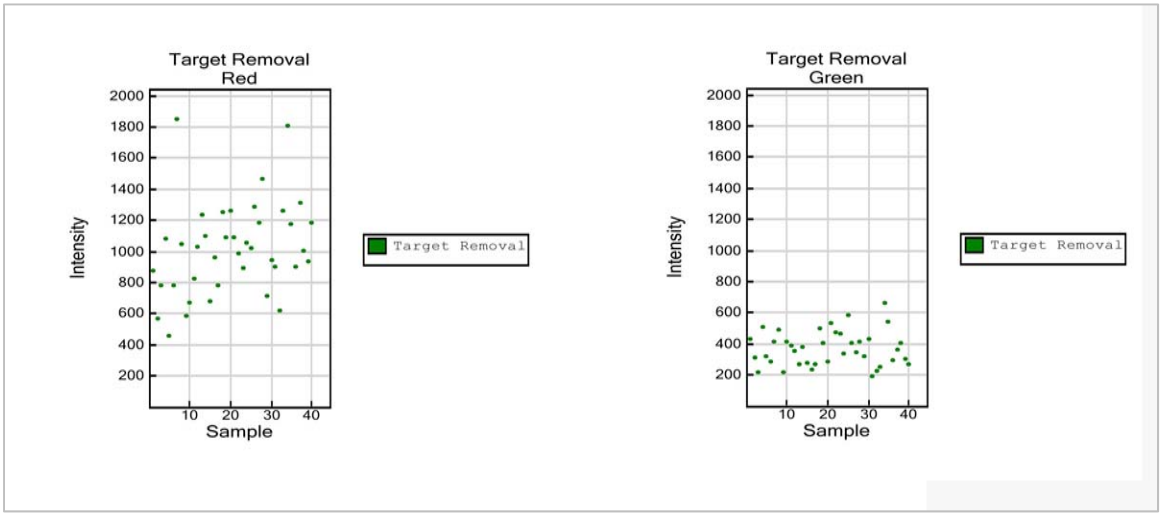


Figure 8: Target Removal Controls

Controls	Expected Outcome
Target Removal Red	Target removal controls are monitored in the red channel. Levels significantly above background in the red channel indicate inefficient target removal.
Target Removal Green	Signal intensity in the green channel is expected to be at background levels.

No maximum threshold value is specified. Please note the difference in scale of the Y axis relative to panels for the Staining Controls and the Extension Controls. Compared to positive signals from the Staining and Extension Controls, data points for the Target Removal Control are expected to be at background levels.

Hybridization Controls (Sample-Independent)

The **Hybridization Controls** assess efficiency of DNA hybridization using synthetic targets instead of amplified DNA. The synthetic targets are added at three different concentrations.

See Appendix A for detailed information for this control.

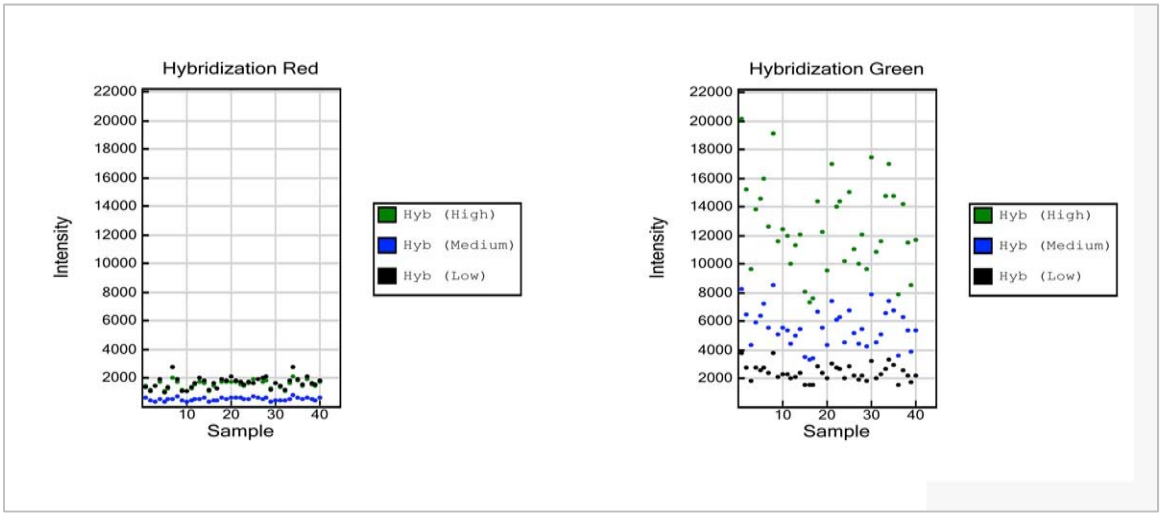


Figure 9: Hybridization Controls

Control	Expected Outcome
Hybridization Red	The Hybridization Controls are monitored in the green channel. Therefore, signal intensities in the red channel are expected to be at background levels.
Hybridization Green	Data points at three different intensity levels are expected, at low (black), medium (blue) and high (green) intensities.

While no threshold values for intensities are specified, a distinct separation of signals by low, medium, and high DNA concentrations within a sample is expected.

Restoration Control (Sample-Independent)

The **Restoration Control** is used to assess the efficiency of DNA restoration in the Infinium HD FFPE protocol.

See Appendix A for detailed information on this control.

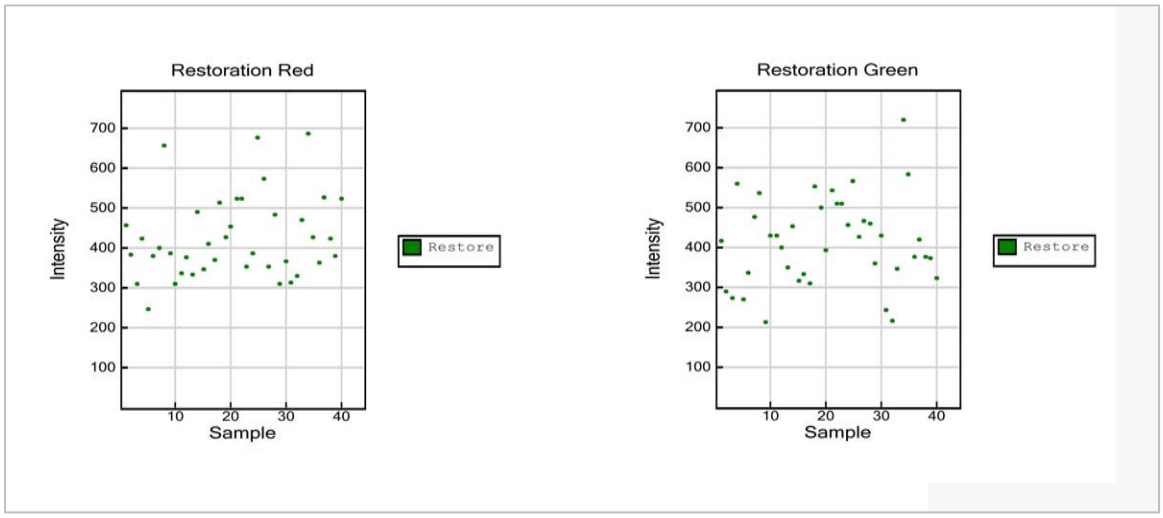


Figure 10: Restoration Control

Control	Expected Outcome
Restoration Red	The restoration control is monitored in the green channel. Therefore, signal intensities in the red channel are expected to be at background levels.
Restoration Green	Strong signal intensity is only expected if samples were treated with Illumina's Infinium HD FFPE Restore Kit. Otherwise, data points are expected to be at background levels.

Stringency Controls (Sample-Dependent)

Perfect Match (PM) and Mis-Match (MM) **Stringency Controls** assess the stringency of the hybridization process.

See Appendix A for detailed information on this control.

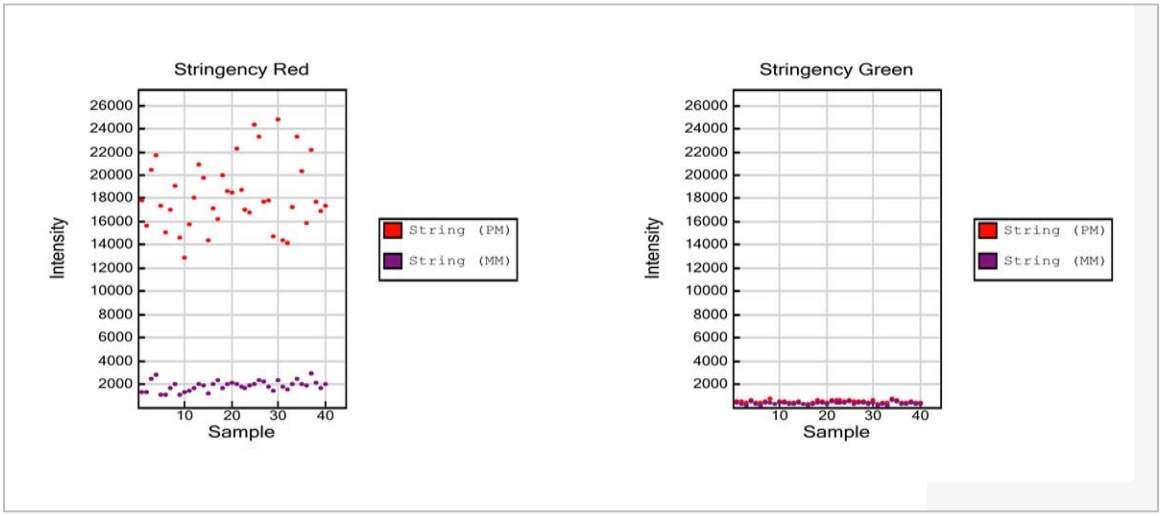


Figure 11: Stringency Controls

Controls	Expected Outcome
Stringency Red	Strong positive signals are expected for String (PM) data points, while String (MM) data points are expected to be at low levels approaching background levels.
Stringency Green	The stringency controls are monitored in the red channel. Accordingly, signal intensities in the green channel for String (PM) and String (MM) data points are expected to be at background levels.

No threshold value for intensities is specified. Stringency controls target human DNA; hence, signals at background levels are expected when running a non-human Infinium assay.

Non-Specific Binding Controls (Sample-Dependent)

The **Non-Specific Binding Controls** test sample quality and specificity of the assay.

See Appendix A for detailed information on this control.

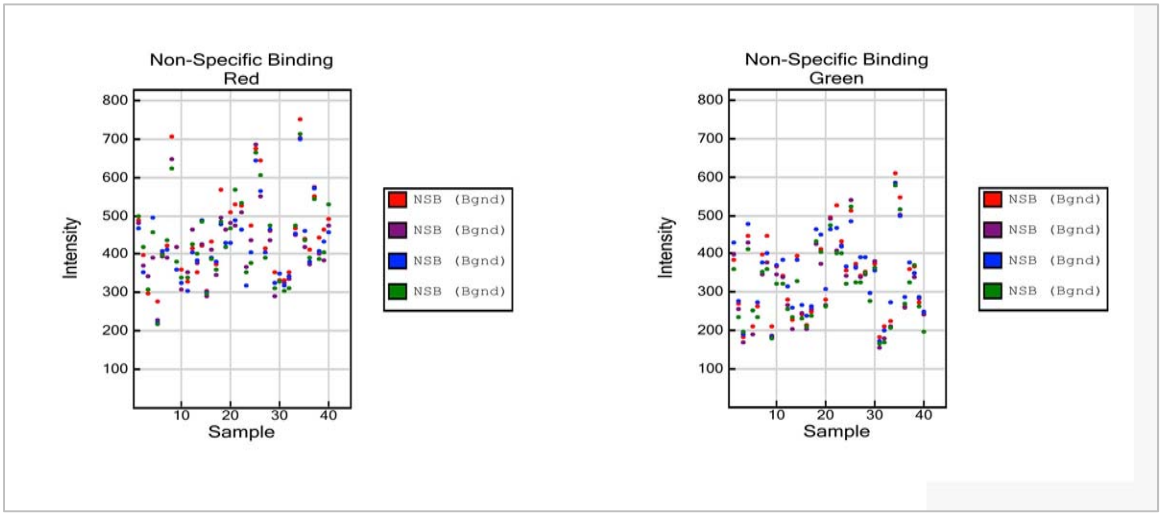


Figure 12: Non-Specific Binding Controls

Controls	Expected Outcome
Non-Specific Binding Red	The probe sequences for Non-specific Binding Controls are complementary to bacterial sequences, and signal intensities at background are expected under standard hybridization conditions.
Non-Specific Binding Green	The probe sequences for Non-specific Binding Controls are complementary to bacterial sequences, and signal intensities at background are expected under standard hybridization conditions.

No maximum threshold value is specified. Please note the difference in scale of the Y axis relative to panels of Stringency Controls and Non-Polymorphic Controls. Compared to positive signals from Stringency and Non-Polymorphic Controls, data points for the Non-Specific Binding Controls are expected to be at background levels.

Non-Polymorphic Controls (Sample-Dependent)

The **Non-Polymorphic Controls** assess sample quality and the overall performance of the assay by querying non-polymorphic regions of the human genome.

See Appendix A for detailed information on this control.

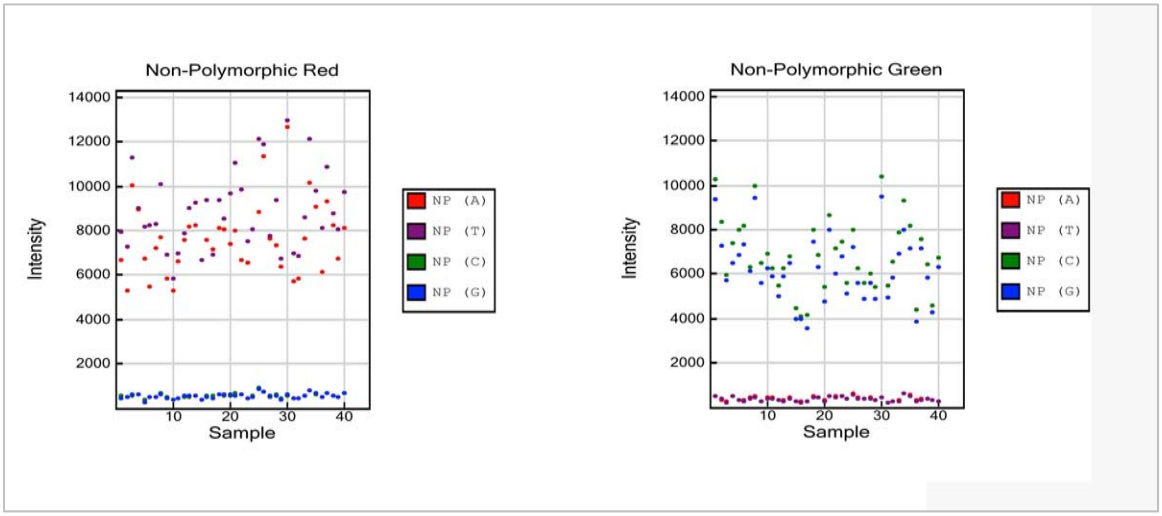


Figure 13: Non-Polymorphic Controls

Controls	Expected Outcome
Non-Polymorphic Red	Strong positive signals are expected for the red NP (A) and purple NP (T) data points, while the green NP (C) and blue NP (G) signals are expected to be at background levels.
Non-Polymorphic Green	Strong positive signals are expected for the green NP (C) and blue NP (G) data points, while the red NP (A) and purple NP (T) signals are expected to be at background levels.

No comparison is made between intensities of positive signals between the red and green channels and A/T and C/G, nor is there a specific threshold value for the intensities. Non-Polymorphic controls target human DNA; hence, signals at background levels are expected when running a non-human Infinium assay.

For More Information

For more information on the Infinium controls and guidelines for how to evaluate them with GenomeStudio, please see the following resources.

GenomeStudio Genotyping Module User Guide
https://support.illumina.com/array/array_software/genomestudio/documentation.html

GenomeStudio Framework User Guide
https://support.illumina.com/array/array_software/genomestudio/documentation.html

Infinium Assay Lab Setup and Procedures
https://support.illumina.com/downloads/infinium-assay-lab-setup-and-procedures-11322460.html

Module Summary

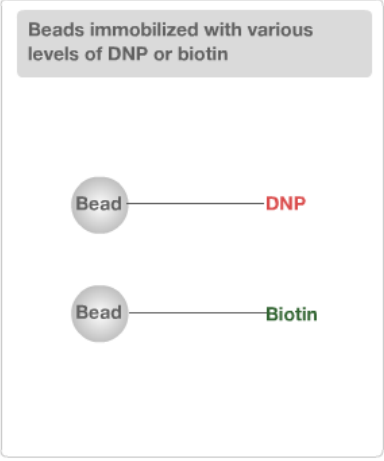
You should now have an understanding of the controls used in the Infinium Genotyping assay, and their expected outcomes. Please take a moment to review the key points from this module.

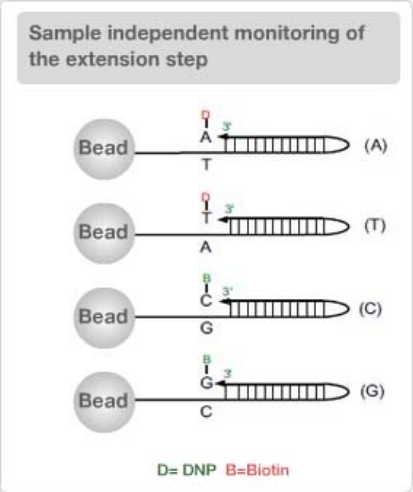
- Infinium controls are designed to support quality control of the assay's stringent performance criteria, and to demonstrate its robustness.
- Sample-dependent controls are Non-Specific Binding, Non-Polymorphic, and Stringency. Sample-independent controls are Hybridization, Extension, Target Removal, Staining, and Restoration.
- View the controls in GenomeStudio by navigating to Analysis > View Controls Dashboard.
- Built-in Infinium controls are most useful when assessed in combination with each other. Evaluate relative (not absolute) intensities of signal to background. Some controls are monitored in one color channel, while others require the evaluation of both red and green color channels.

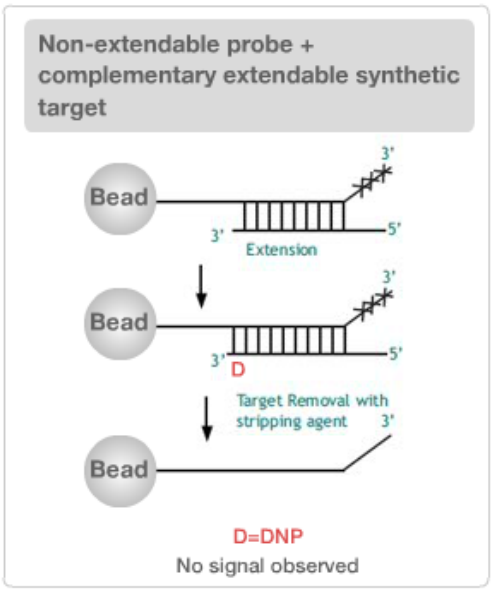
APPENDIX A

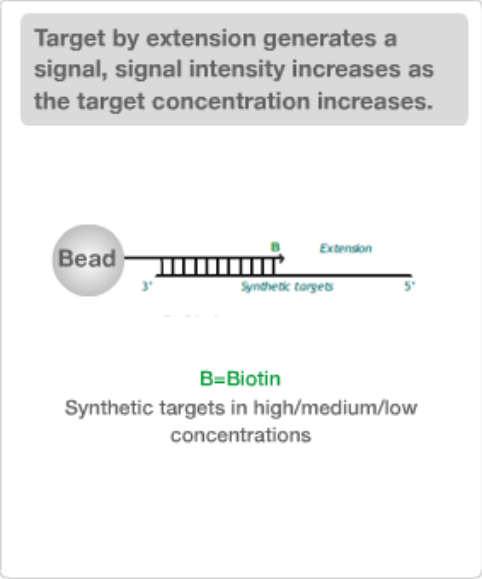
Infinium Controls in Detail

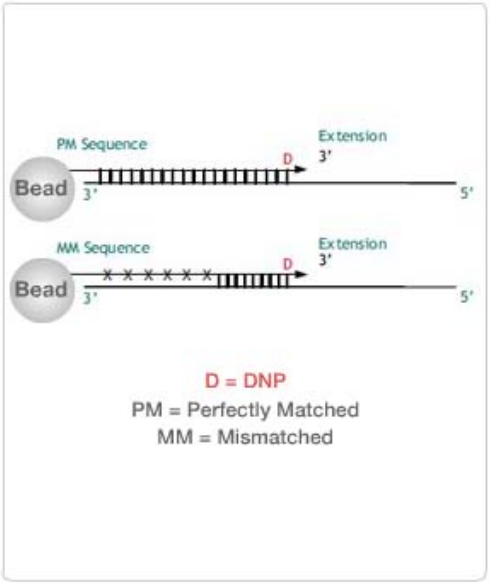
This appendix provides more information about the Infinium controls and bead types.

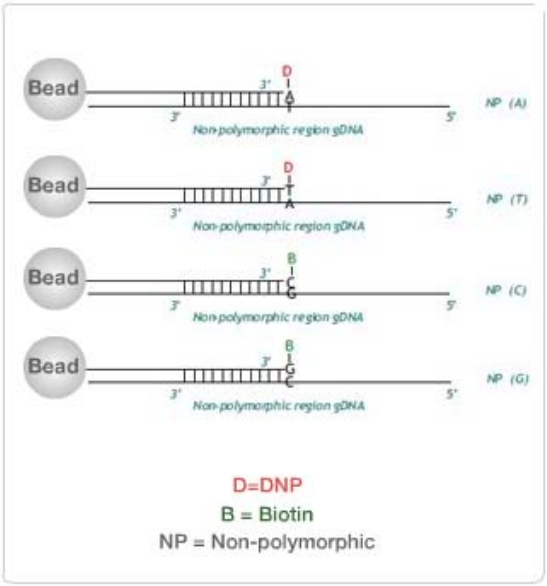
Term	Definition
Sample-independent controls	Sample-independent controls are used to evaluate chip performance and are informative of efficiency of hybridization and staining. Sample-independent controls cannot be used to assess sample quality or to identify issues with sample processing. Even if highly degraded DNA was hybridized to a chip, sample-independent controls are expected to appear normal, given that all steps preceding hybridization were performed according to protocol. This is because DNA targets for sample-independent control probes do not originate from the sample DNA. Target Removal Controls and Hybridization Controls probe targets are spiked into the hybridization buffer. The sample-independent controls include Staining Controls, Extension Controls, Target Removal Controls, and Hybridization Controls. The Restoration Control can also be categorized as sample-independent, since it does not depend on sample quality. However, the Restoration Control is only meaningful when samples were processed with the FFPE Restore kit prior to the Infinium assay.
Staining Controls *Sample-independent control*	Staining Controls are used to examine the sensitivity and efficiency of the staining step in the X-stain. Staining controls consist of beads covered with high levels or small (background) levels of dinitrophenyl (DNP) or biotin, and are directly labeled in successive rounds of adding green fluorescent streptavidin and red fluorescent anti-DNP antibody. Because DNP and biotin are directly attached to the beads, staining controls do not depend on DNA hybridization to the chip and do not require single-base extension. Staining Controls are monitored in both the red and green channels. 

Term	Definition
Extension Controls *Sample-independent control*	Extension Controls test the efficiency of single base extension during the X-stain. Extension Controls consist of hairpin oligos that function as both template and probe. During X-stain, probes are extended at the 3' end using the probe strand itself as a template. Accordingly, extension controls are independent of DNA hybridization, but require successful single base extension and staining. Both red (extension with A or T) and green (extension with C or G) channels are monitored. 

Term	Definition
Target Removal Controls *Sample-independent control*	Target Removal Controls test the efficiency of stripping off DNA template after the extension reaction. The probe sequences are designed such that extension from the probe does not occur. Instead, the control target DNA, added with the hybridization buffer, is extended and labeled using the probe sequence as templates. Target Removal controls should yield signal intensities at background levels. Failure to efficiently remove target DNA will result in an increase in signal intensities over background levels. Target Removal Controls are monitored in the red channel. 
Hybridization Controls *Sample-independent control*	Hybridization Controls test assay performance using synthetic targets instead of amplified DNA. These synthetic targets bind to complement probes on the array, and provide templates for single base extension. The synthetic targets are present in the hybridization buffer at three levels, monitoring the response from high-concentration (5 pM), medium concentration (1 pM), and low-concentration (0.2 pM) targets. Hybridization Controls appear in the green channel as signals with various intensities, corresponding to the concentrations at which targets are spiked into the assay. Hybridization Controls require optimal stringency conditions during hybridization and washing of BeadChips, and they depend on successful single base extension and staining.

Term	Definition
	
Restoration Control *Sample-independent control*	The Restoration Control is specific to the Infinium HD FFPE Assay that requires formalin-fixed paraffin- embedded (FFPE)-derived DNA to be restored using Illumina's Infinium HD FFPE Restore Kit. The Restoration Control does not depend on the quality of sample DNA. It should show no activity for samples that have not been processed with the FFPE Restore kit. The Restoration Control uses a short oligo that has been spiked into the Infinium HD FFPE restore kit. Due to the chemistry of the Infinium HD FFPE Restore Kit, the control oligo will be available to bind to its complement on the BeadChip only if the restore process functions properly. Detection of reduced intensity of the Restoration Control may indicate that the DNA restoration process has been compromised. This control is detected in the green channel.
Sample-dependent Controls	The sample-dependent controls are used to evaluate assay performance across samples, and assess sample DNA quality. With the exception of Non-Specific Binding Controls, sample-dependent controls are specific to human DNA targets and cannot be used for interpretation of data obtained with non-human Infinium products, including non-human iSelect chips. In addition to poor sample quality, failure of any step in the assay (amplification, hybridization, X-stain) will result in depressed sample-dependent controls. Sample-dependent controls include Stringency Controls, Non-Specific Binding Controls, and Non-Polymorphic Controls.

Term	Definition
Stringency Controls *Sample-dependent control*	Stringency Controls test the stringency of the hybridization process. High stringency is achieved through increased temperature and optimized composition of the hybridization buffer. Perfect match (PM) controls are exactly complementary to their human DNA target, resulting in high signal intensities. In contrast, mismatch (MM) controls probes have mismatched nucleotides between target and probe to affect hybrid stability, and are expected to yield signals of much lower intensity under optimal stringency conditions. Performance of Stringency Controls is monitored in the red channel. 
Non-specific Binding Controls *Sample-dependent control*	Non-Specific Binding Controls are included to monitor the specificity with which amplified DNA hybridizes to the chip, assess sample DNA quality, and identify presence of non-human DNA. The probe sequences for Non-specific Binding Controls are complementary to bacterial sequences and should not hybridize to human sequences under standard hybridization conditions. Loss of the specificity of the assay and binding of non-human sequences complementary to control probes are expected to lead to increased signal intensities. These controls are monitored in both, the green and red channel, and should show intensities at background levels in both.
Non-polymorphic Controls *Sample-dependent control*	Non-Polymorphic Controls test the overall performance of the assay, from amplification to detection, by querying a particular base in a non-polymorphic region of the human genome. Non-polymorphic Controls are used to compare assay performance across different samples. One non-polymorphic control has been designed for each of the four nucleotides. A and T are monitored in the red channel, and C and G in the green channel.

Term	Definition
	

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