Best Practices for Infinium® Assays

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Introduction

This document contains the latest Illumina-supported improvements and best practices for the Infinium Assay. It is a supplemental guide to the commercially released Infinium Assay protocols, designed to optimize sample processing in the varying lab conditions of Illumina’s customers worldwide. Revisions to this document will be made in the event of additional recommendations.

Evaluate Data in GenomeStudio First

When evaluating assay performance, it is important to analyze data in Illumina’s GenomeStudio data analysis software before inferring the impact of any observation on overall data quality. For example, sample evaporation around the loading port on a BeadChip is associated with decreased intensity. However, observed sample evaporation during BeadChip processing may not ultimately have a significant impact on data quality. In addition, it is important to note that decreased intensity on areas of the array away from the sample loading port does not correlate with sample evaporation. It is essential to conduct an investigation in GenomeStudio before drawing any conclusions about assay performance.

Reference Material

Please refer to the following documentation for complete instructions on assay protocols. Electronic copies of the latest documentation are available at http://www.illumina.com/documentation. An iCom login is required.

Infinium HD Ultra Assay

- Infinium HD Ultra Assay Guide (PN 11328087)
- Infinium HD Ultra Assay Experienced User Card for Automated Processing (PN 11328108)

Infinium HD Super Assay

- Infinium HD Super Assay Protocol Guide (PN 11322427)
- Infinium HD Super Assay Experienced User Card for Automated Processing (PN 11322291)

Infinium HD Gemini Assay

- Infinium HD Gemini Assay Guide (PN 11311007)
- Infinium HD Gemini Assay Experienced User Card for Automated Processing (PN 11311023)
- Infinium HD Gemini Assay Experienced User Card for Manual Processing (PN 11311015)
We are always interested in hearing how other members of the Illumina Community are optimizing their research. If you would like to share your methods for getting the most out of the Infinium Assay, please contact Illumina Technical Support. If you have any questions about this document or about how to implement the best practices it describes, contact Technical Support or your local Field Applications Scientist.

### Table 1  **Illumina Customer Support Contacts**

<table>
<thead>
<tr>
<th>Contact</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toll-free Customer Hotline</td>
<td>1-800-809-ILMN (1-800-809-4566)</td>
</tr>
<tr>
<td>International Customer Hotline</td>
<td>1-858-202-ILMN (1-858-202-4566)</td>
</tr>
<tr>
<td>Illumina Website</td>
<td><a href="http://www.illumina.com">http://www.illumina.com</a></td>
</tr>
<tr>
<td>Email</td>
<td><a href="mailto:techsupport@illumina.com">techsupport@illumina.com</a></td>
</tr>
</tbody>
</table>
Optimizing Intensity Around the Loading Port

The practices described in this section have been found to reduce the incidence of decreased intensities on Infinium BeadChips around the loading port.

Check Z-Height on Robot Deck

Step in Assay
- All steps that involve the Tecan robot, and especially the Hybridize BeadChip step

Best Practice

Review the plate's sample volumes throughout the protocol to ensure that the Tecan robot is dispensing and aspirating correctly to all wells.

If you encounter any problems with sample evaporation, check the Z-height of the tips on the Tecan robot and verify the amount of sample remaining in the amplification plate. It may be that the Tecan robot is underloading samples. If you suspect that the Tecan tip height is out of specification, contact an Illumina Field Service Engineer.

Background Information

Correct tip height is an important component of good assay performance. If the robot tips are too high and far away from their optimized position within the wells of the sample plate, then the robot cannot aspirate the required amount for sample hybridization. If tip height is too high during sample loading, for example, then less sample will be dispensed onto the BeadChip. If significant enough, underloading can lead to sample evaporation during hybridization that can affect data quality.

If the tip height is too low and the tips bottom out in the sample well, this can also impair proper aspiration of the plate contents, by impeding the tip’s ability to draw up the correct sample volume.

Use Fresh RA1 for Each Step

Step in Assay
- Resuspend the MSA/AMP Plate
- Single-Base Extension and Stain HD BeadChip (XStain)

Best Practice

The RA1 reagent is used in the Resuspension and XStain steps of the Infinium Assay. RA1 is shipped frozen and must be gradually warmed to room temperature, then mixed gently to dissolve any crystals. RA1 that has been stored properly and has not been dispensed for use in either the XStain or Resuspension step is considered fresh RA1. It is important to use fresh RA1 for each protocol step in the assay where it is required. This is especially critical for the resuspension step.

After RA1 has been poured out into a reservoir and exposed to room temperature air for extended periods of time, it is no longer fresh.
To make best use of RA1, only pour out the amount needed for the current step. If you plan to perform additional assay steps requiring RA1 that same day, then leave the remaining thawed reagent in the original, closed bottle at room temperature until it is needed. Otherwise, follow the standard RA1 storage procedures described in the assay guide for next-day processing and prolonged storage conditions.

**Background Information**

Figure 1 shows results from a controlled experiment done to measure the changes in the osmolality of RA1 over prolonged exposure to specific environmental conditions. The experiment took place over an 8-hour period where a half-reservoir of the reagent at various fill volumes was exposed to a 12% relative humidity environment. The data points are extrapolated from osmometer measurements for varying volumes of RA1. The graph in Figure 1 shows a line for each reservoir fill volume: 10 ml, 15 ml, 20 ml, 25 ml, and 30 ml.

![Figure 1 Time Series Model of RA1 Evaporation at RT](image)

The X-axis represents elapsed time in hours, and the Y-axis represents the theoretical formamide+salt concentration as a total percent. The graph clearly indicates that prolonged exposure of RA1 over the course of a day increases the overall concentration of formamide+salt in the reagent solution as water is lost through evaporation. The effect is more pronounced for lower starting volumes in the reagent reservoir.

**Step in Assay**

- Hybridize Multi Bead Chips | Assemble the Hyb Chambers

**Best Practice**

1. After you fill the Hyb Chamber reservoirs with PB2, place the lid on the Hyb Chamber right away to prevent evaporation. The lid does not need to be locked down.

2. After the Hyb Chamber has been loaded with PB2, load BeadChips into the Hyb Chamber within one hour.
Background Information

Changes to the osmolality of PB2 shift the vapor pressure balance between RA1 (sample hyb buffer) and PB2 away from its optimized point. An imbalance in the vapor pressure between RA1 and PB2 can lead to increased sample evaporation during the hybridization process. As the data in Figure 3 show, the osmolality of PB2 changes at a much faster rate if the chamber lid is left open.

Figure 3 compares the evaporation rate of PB2 in closed Hyb Chambers versus open Hyb Chambers at room temperature with 37% relative humidity. The PB2 was serially loaded into Hyb Chambers at 2 hours, 1.5 hours, 1 hour, and 0.5 hours before sample loading. One chamber was left open throughout, and the other one was closed after each addition.

The Y-axis shows the increasing range of osmolality (measure of osmoles of solute per kilogram of solvent), as measured by an osmometer when the PB2 was left exposed to the lab environment.
Cool plate for 30 minutes
After Denaturing

Step in Assay
- Hybridize Multi Bead Chips | Hybridize Sample to Multi BeadChip

Best Practice
1. After the 20-minute incubation at 95°C, remove the amplification plate from the heat block and place it on the lab bench.
2. Leaving the seal intact, cool the plate at room temperature for 30 minutes.
3. Continue with the instructions in the assay guide, beginning by pulse centrifuging the plate to 280 xg.

Figure 4  Denaturation Step in Infinium Assay

Background Information
Figure 5 compares the water loss between two amplification plates. After the 20 minute incubation at 95°C, the seal was removed from one plate, and then both plates were left to cool at room temperature for 30 minutes. The plate weight was measured every five minutes. Cumulative water loss per sample was calculated based on the weight loss of the entire plate over that period. The unsealed plate lost approximately 9 μl water per sample, while the sealed plate lost less than 1 μl.

Figure 5  Water Loss From Sealed and Unsealed Plates

These data show that sample evaporation can be caused by exposing the heated samples to the lab environment immediately after denaturation. The loss of sample volume from the plate wells can lead to lower sample hybridization volumes on the BeadChip. Underloading the BeadChips makes them more susceptible to lower intensity areas caused by evaporation.
It is important to note that the majority of the sample volume loss occurs within the first 10 minutes after removing the plate seal. Letting the sample plate cool for 30 minutes on the lab bench allows the seal to capture the condensate volume that would have evaporated had the plate seal been immediately removed. Pulse centrifuging the plate after cooling it down draws the condensate back into the sample well.

**Do Not Precondition Hyb Chambers**

**Step in Assay**
- Hybridize Multi Bead Chips | Load BeadChip

**Best Practice**
Hyb Chambers should be at room temperature when you load the BeadChips. They should not be preconditioned in the Illumina Hybridization Oven prior to loading the BeadChips. Heating the PB2 and then opening the Hyb Chamber to add BeadChips causes some of the PB2 to evaporate, leading to a change in the osmolality of PB2 and an imbalance in the vapor pressure between PB2 and RA1 (sample hyb buffer).

**Check Loading Port for Bolus of Liquid**

**Step in Assay**
- Hybridize Multi Bead Chips | Load BeadChip

**Best Practice**
This best practice applies only to manual loading. Any loading problems that occur during robotic loading should be referred to an Illumina Field Service Engineer.

1. After loading all DNA onto the BeadChip, wait for the sample to disperse over the entire surface.

2. Inspect the loading port to see if a large bolus of liquid remains. Excess sample volume in the BeadChip loading port helps prevent low-intensity areas resulting from evaporation.

*Figure 6  Loading BeadChip with Sample*
3. If no excess liquid is visible, it is acceptable to add additional sample from the leftover volume in the amplification plate until there is a large bolus around the loading port.

4. Record the top-off activity on the lab tracking form.

CAUTION Do not top off with RA1 (sample hyb buffer), as this will dilute the sample.

Do Not Modify the Hybridization Environment

Step in Assay
- Hybridize Multi Bead Chips
- Set up Multi BeadChip for Hyb

Best Practice
After loading the BeadChips into the Hyb Chambers, place the Hyb Chambers into the Illumina Hybridization Oven immediately. Do not modify the hybridization environment by adding additional fixtures or humidifying elements. Leave the Hyb Chambers in the oven at the correct orientation and temperature until hybridization is complete. Changes to the hybridization environment can have unexpected effects on data quality.
Optimizing Intensity in Other Areas of the BeadChip

The practices described in this section have been found to reduce the incidence of decreased intensities in areas that are not around the sample loading ports of the BeadChips.

Cool Hyb Chamber for 25 Minutes After Incubation

Step in Assay
- Wash BeadChip

Best Practice
1. After removing the incubated Hyb Chamber from the Illumina Hybridization Oven, leave the chamber with the lid closed on the benchtop at room temperature for 25 minutes.
   For efficiency, use the 25 minutes to prepare wash dishes, spacers, and glass back plates for washing.
2. After this cooling period, follow the instructions for removing the seals and washing the BeadChips.

![Figure 9 Step in Assay](image)

Background Information

Internal Illumina studies have shown that allowing BeadChips to cool down before removing the seals reduces the occurrence of dim areas on the side of the BeadChip where the IntelliHyb seal was first pulled off.

Remove BeadChip IntelliHyb Seal Slowly

Step in Assay
- Wash BeadChip

Best Practice

When removing the seal from the BeadChip, slowly creep the seal diagonally along the surface of the BeadChip until the first corner of the sample area on the BeadChip is exposed. Continue peeling off the seal in a slow and steady motion until it is completely removed. Immediately place the peeled BeadChip into wash buffer.

It is especially important at the beginning of the seal removal process that the seal adhesive is broken away slowly from the BeadChip surface. This technique is most effective when preceded by the 25 minute cooldown period after incubation.
Background Information

If the IntelliHyb seal is removed too quickly, the corners of the stripes at the bottom of the sample section where you first removed the seal may display decreased intensity. In severe cases, there may be negative effects on call rates and logRdev. Slow, deliberate removal also helps ensure that the glue does not remain on the surface of the BeadChip. Residual glue can interfere with the effectiveness of the XStain process to a greater or lesser degree, depending on the location and severity of the residue.

General Best Practices

The following best practices have not been formally tested for correlation with intensity, but have been found to ensure better results in the assay.

Mix Reagents Thoroughly

Step in Assay

- All Steps

Best Practice

It is important that all samples are completely thawed and well mixed before being used in the assay. After thawing reagents to room temperature, always invert them 10 times and pulse centrifuge them to 280 xg for 1 minute.

Keep Hyb Chamber Lids and Bases Together

Step in Assay

- Hybridize Multi Bead Chips I Assemble the Hyb Chambers

Best Practice

To ensure optimal results from Hyb Chambers:

- Adopt a labeling convention that keeps each Hyb Chamber base paired with its original lid.
- Check Hyb Chamber lid-base pairs regularly to ensure that the fit remains secure.
Check hinges regularly for any signs of abnormal wear or loose fittings. It is important that the hinges provide adequate clamping strength to ensure an airtight seal between the lid and the base.

Record the Hyb Chamber that was used for each BeadChip, so that Hyb Chambers can be investigated and evaluated in the event of sample evaporation or other lab processing anomalies.

**Do Not Replace PB2 with RA1**

**Step in Assay**
- Hybridize Multi Bead Chips | Assemble the Hyb Chambers

**Best Practice**

Do *not* replace PB2 in the Hyb Chamber with RA1. This will decrease the stringency and may negatively affect sample call rates and logRdev. PB2 is formulated to produce the appropriate amount of humidity within the Hyb Chamber environment to prevent sample from evaporating during hybridization.

**Place BeadChip in PB1 Immediately After Removing Seal**

**Step in Assay**
- Wash BeadChip

**Best Practice**

1. After removing the IntelliHyb seal from the hybridized BeadChip, immediately place the BeadChip into PB1.

**CAUTION**

Do not attempt to remove any glue residue from the BeadChip surface at this time.

![Figure 11 Placing BeadChip Immediately into Wash Rack with PB1](image)

2. Repeat this for each BeadChip until they are all placed in the PB1.

3. Ensure that all BeadChips have been in the PB1 buffer for at least one minute before proceeding.

This neutralizes the BeadChip surface in order to maintain high data quality and assay performance.
Step in Assay

Remove Seal Residue under PB1

- Wash BeadChip

Best Practice

1. After all the BeadChips have been in PB1 for at least one minute, place them into the Alignment Fixture as directed.

2. Before placing the spacer on top of the BeadChips, inspect the surface of each BeadChip for residue left by the seal.

3. If you see any, carefully use a pipette tip to remove any residue under buffer. Be very careful not to scratch the bead area.

4. When the BeadChips are free of residue, proceed to place the spacers and assemble the Flow-Through Chambers as instructed.

Disassemble Flow-Through Chambers Immediately After XStain

Step in Assay

- Stain BeadChip

Best Practice

It is important that BeadChips are not allowed to dry after staining.

1. Remove the Flow-Through Chambers from the Chamber Rack immediately after staining and place them horizontally on a lab bench at room temperature.

2. Disassemble the Flow-Through Chambers and submerge the BeadChips in the wash dish as soon as possible.

Stain BeadChip

1. If you plan to image the BeadChip immediately after the staining process, turn on the Illumina BeadArray Reader now to allow the lasers to stabilize.

2. Into the reservoir of each Flow-Through Chamber, dispense:
   a. 250 µl STM and incubate for 10 minutes.
   b. 450 µl XC2 and incubate for 1 minute. Repeat once, and then wait 5 minutes.
   c. 250 µl ATN and incubate for 10 minutes.
   d. 450 µl XC2 and incubate for 1 minute. Repeat once, and then wait 5 minutes.
   e. 250 µl STM and incubate for 10 minutes.
   f. 450 µl XC3 and incubate for 1 minute. Repeat once, and then wait 5 minutes.
   g. 250 µl ATN and incubate for 10 minutes.
   h. 450 µl XC3 and incubate for 1 minute. Repeat once, and then wait 5 minutes.
   i. 250 µl STM and incubate for 10 minutes.
   j. 450 µl XC3 and incubate for 1 minute. Repeat once, and then wait 5 minutes.

2. Immediately remove the Flow-Through Chambers from the Chamber Rack and place horizontally on a lab bench at room temperature.

Figure 12 Stain BeadChip Step in Assay