

Protein Detection

1. How does the GXII determine protein concentration?

Protein samples contain a single lower marker. Alignment to a single marker does not provide enough constraints to align large proteins, so data is aligned to two ladders, one sipped just before the sample wells and another sipped just after the sample wells. Samples are scaled so the sample's lower marker is nearly aligned with both ladder lower markers. The scaling is weighted by the proximity in sip time to each ladder. The sample sipped closest to the primary ladder is scaled to align more closely to the primary ladder and the sample sipped just before the bracket ladder is scaled to align most closely to the bracket ladder. Then each sample is shifted in time so the sample's lower marker aligns exactly with the primary ladder lower marker. After alignment, the size of the protein producing each peak is calculated from the aligned peak time using a log (size) versus 1/Time fit to the primary ladder peaks of known size and measured migration time. This fit to the ladder peak data can be viewed by selecting Standard Curve from the Analysis Menu.

To determine sample peak concentration, the peak areas are first corrected to compensate for the fact that the fluorescence intensities are sampled at a constant time interval so slower moving proteins spend more time under the detector than fast moving proteins. The peak concentration is then calculated using the ladder peak areas and concentration for the ladder supplied in the Assay Analysis window. The concentration is adjusted for the differing dilution ratios of sample and ladder.

2. How can I use the information from the protein standard curve to adjust the GX protein concentrations?

To quantify the sample peak concentration based on the protein standard curve, the new standard must be added into each sample well at a known concentration. The analysis settings provide a Sample Peak Quantitation option using the peak area and concentration of the User Standard instead of the ladder concentrations.

Users may label their protein at a single high concentration and perform the serial dilution. However, the results obtained at any lower dilution may not represent the actual labeling efficiency if a user actually started at that dilution.

Alternatively, users can serially dilute their protein and label each dilution. Although this standard curve will contain more noise, it will be a better representation of actual labeling efficiency.

We recommend that at very high or very low protein concentrations, dye to protein ratios be increased above 1:15 for maximum labeling efficiency. At low protein concentrations, the kinetics may require longer incubation times which may be undesirable to the user. Higher dye concentrations will mitigate this issue. At very high protein concentrations, more dye molecules are required to label all available sites on the protein.

A general rule of thumb is to use 20uM dye at reaction or 1:15 protein/dye ratio, whichever results in the greater amount of dye at any given protein concentration. It is also recommended that the concentration of DMSO included in each reaction be kept constant since this might also influence labeling efficiency. It is recommended that the amount of DMSO be no higher than 10% of the labeling reaction.

3. What is the optimal protein concentration for labeling?

Frequently Asked Questions

The recommended optimal protein concentration range for labeling is 100ng/μL to 1000ng/uL, although lower protein concentrations can also work. It is important to note that the protein to dye ratio should remain constant if protein concentration is to vary.

4. What is the optimal protein to dye ratio?

The optimal protein to dye ratio will vary from protein to protein. However, Caliper has found that the range of optimal ratios typically falls between 1:6 to 1:15. It is recommended that users find the optimal ratio for their protein of interest. When labeling very high or very low protein concentrations, users may have to increase the amount of dye to protein used. As a quick rule of thumb: use 20uM of dye in the labeling reaction or a 1:15 ratio, whichever is greater. See question 2 for a related question.

5. Can one manually override the quantification and use known protein standard values?

Yes. LabChip GX Version 2.1 software supports a user defined standard curve to improve concentration estimates.

6. Do I have to worry about carryover on the pico protein chips?

The specification for well to well signal carryover on the pico protein kit is >0.5%. So unless the Ab starting concentration is greater than 10ng/μL or 2ng/μL for proteins, carryover should not be a problem. For proteins above these concentrations, user should follow high protein wells with a no protein well before the next sample well is read on the plate.

7. Is there a way to analyze proteins below 14kDa?

Removal of free dye may allow users to analyze proteins below the kit specifications. Please refer to the "Removing Free Dye from Samples" in the alternate preparation procedure section to quantify proteins less than 15kDa range.

8. What is the lowest level of contamination detectable on the kit?

The kit is able to detect down to 0.1% contamination of protein. This is based on a protein starting concentration of 400ng/uL.

9. Can you detect aggregates of antibodies?

Yes. Covalent aggregates can be detected. If users wish to determine the level of covalent aggregates held together by disulfide bonds, users will have to run both nonreduced and reduced protocols. Generally, higher protein concentrations will aid in the detection of aggregates since populations tend to represent only a small percentage of total sample being analyzed.

10. Can you detect antibody glycosylation?

Yes, we can distinguish between glycosylated and non-glycosylated heavy chain, and determine glycan occupancy. Ask about Caliper's upcoming Glycan Profiling kit which will profile antibody glycosylation in a high throughput format.

Kit Specification

1. What are the storage conditions for the various kit components?

Chips and reagents are to be stored refrigerated at 4°C. Reconstituted dye, if not used within 1 week, should be kept at -20°C. Short term storage of the dye is at 4°C. Chips can be stored at room temperature for up to 30days. Long term storage should be at 4°C.

2. How stable is the reconstituted dye and how can I extend the life of the dye?

The reconstituted dye has demonstrated stability up to 2 years at -20°C. However, the dye may be kept at 4°C for shorter period as it is being used in the short term. Once the dye is reconstituted with water, it is not considered stable. Dye reconstituted in water should not be kept for periods longer than an hour.

3. My protein seems to require higher than normal dye to protein ratios. I am running out of dye before the rest of the reagents in the kit are being used up. Can I purchase the dye separately?

As of yet, there is no plan to sell the dye separately. However, users should contact their Caliper representative if there is interest in purchasing the dye individually.

4. Why must the 10M NEM be made fresh each day?

NEM reconstituted in DMSO is not considered stable and must be made fresh each day. Nonreduced antibodies may fragment when using old 10M NEM.

5. What is the shelf life of the kit and its components?

6 months.

6. Can the kit be used in a Code of Federal Regulation (CFR) environment?

Yes. Caliper has CFR software that can run in the background of the pico protein assay.

7. What kit components must be provided by the user and why?

Users are asked to provide their own molecular biology grade water, 70% isopropanol, 1M DTT, analyte buffer and 10M NEM in DMSO. The first two components are meant to reduce overall cost of the kit. DTT must be shipped at -20°C and is thus not included. It is preferable that users should use their own analyte buffer but should abide by the recommended pH conditions. The alkylating agent is unstable when dissolved and is thus not included in the kit.

8. How stable is NEM once it has been reconstituted with the labeling buffer?

N-ethylmaleimide is unstable at RT after reconstitution in DMSO, ethanol or water and must be used within an hour. Unused 10M NEM should be discarded at the end of each day. However, aliquots of 10M NEM in DMSO can be kept frozen at -20°C for single use only.

9. What proteins does Caliper use to validate the linear concentration range of its kit?

Caliper uses a monoclonal antibody and non-acetylated Bovine Serum Albumin to verify the kit specifications. Caliper uses Bovine Albumin (Fraction V Powder, Fatty acid free, low endotoxin) from Sigma, Catalogue number A-8806. Labeling efficiencies will vary from protein to protein, depending on buffer compatibilities, pH dependences and the number of lysine residues available for labeling.

10. Why doesn't Caliper have specifications on the accuracy of its protein quantification?

The quantification of proteins in the Pico Protein Express LabChip Kit is based on comparison against a standard protein ladder. There is no guarantee that the labeling of Caliper's protein ladder will reflect the efficiency of labeling for any particular protein a user might be investigating. Generally, it is best to prepare a standard protein ladder for better quantification.

Assay Preparation**1. How long does sample prep take?**

Generally, a sample prep procedure should take no more than 2 hours before the plate is ready by the GXII instrument. This will vary depending on the number of samples and the complexity of the experiment.

2. What protein buffers are compatible with this assay?

There is an extensive list of compatible buffers, salts and additives for this assay. Please refer to the chart at the back of this manual under LabChip Kit Essential Practices for more information. However, it is important to note that there are pH recommendations for use in this kit. If a user is unsure if a particular buffer is compatible with labeling, one can always perform a buffer exchange with the buffers provided in the kit.

3. What are the guidelines for buffer exchange?

Caliper recommends that users use centrifuge type columns and perform three exchanges into the buffer of choice. Each exchange should be in a 1:10 dilution of the original buffer. The original buffer should not be a higher molarity than the final buffer.

4. How long does it take to run 12 samples or 96 samples on the GXII?

After the chip and samples have been prepared, to run 12 samples takes approximately 30 minutes and to run 96 samples takes about 80 minutes. The processing time is less than 50 seconds per separation.

5. Can you process both denatured/reduced antibodies as well as nonreduced antibodies on the 96 well plate?

Yes. The general protocol can be used to process both reduced and nonreduced samples on the same plate. See question 1 above.

6. My protein seems to be fragmenting during the nonreduced protocol. How can I better protect sensitive antibodies or chimeric antibodies?

Sensitive antibodies or proteins may require a more gentle labeling buffer and or procedure. If you discover that you may require that, follow the Preparing nonreduced samples for sensitive antibodies procedure under Alternate Preparation Procedure section.

7. The lyophilized dye pellet seems smaller than usual when I reconstituted it. Should I continue using it?

Occasionally, a portion of the dye pellet may not enter the vial during manufacturing. If you notice your reconstituted dye is either clear or noticeably lighter in coloration, please contact Caliper.

8. I have a protein sample with an unknown concentration. Which protocol should I use?

It is best to prepare a protein standard curve to quantify an unknown. However, if the sample is limited, then we would recommend the Labeling Protein of Unknown Concentration protocol. If the signal saturates on the GX, users will have to dilute the sample to analyze their protein sample.

9. If I am using your kit for the first time, how do I confirm that the kit is working properly?

Users may confirm kit performance by labeling commercially available non-acetylated bovine serum albumin. See question 9 in the kit specification section.

10. Can I use TCEP (Tris(2-Carboxyethyl) phosphine) instead of DTT to reduce my protein?

Caliper does not recommend use of TCEP to reduce proteins.

11. What steps should I take to optimize the kit for my protein(s) of interest? How many proteins can I optimize in one kit?

Users can follow the general protocol for reduced or nonreduced proteins as a first step. If the results match well against orthogonal methods, no further optimizations are necessary.

If labeling efficiencies seem low under reduced conditions for antibodies, users should try to increase dye to protein ratio, protein concentration or length of labeling. As a quick first step, users may want to try the Labeling Reduced Antibodies for HC/LC analysis. This method incorporates higher concentrations of protein and dye for optimal labeling. However, individual results may vary and further optimizations may be required.

If protein samples seem to be fragmenting in nonreduced conditions, try lowering the 75°C incubation time from 5 minutes or decreasing the temperature. Alternatively, try the Labeling nonreduced samples for sensitive antibodies. However, this non-high throughput method deviates from the general protocol and cannot be used in conjunction with the reduced protocol on the same plate. If you suspect that the protein is under labeled, try increasing the dye to protein ratio, protein concentration or length of labeling to more than an hour.

Depending on the stability of the protein, the number of available sites for labeling and other characteristics of a particular protein, the number of optimizations will vary. Generally, users should be able to optimize for several proteins with one kit.

Frequently Asked Questions

12. What's the difference between using the General Protocol for Reduced Protein and the Labeling Reduced Antibodies for HC/LC analysis?

The general protocol uses a lower concentration of protein and dye than the HC/LC specific protocol. The dye in the general protocol is reduced to 40uM by the addition of water. This dye dilution step maintains the protein to dye ratio at 1:15 when the protein starting input is 0.4mg/mL. The general protocol also avoids adding dye in volumes less than 5uL. It also minimizes dye usage by diluting the dye but users should keep in mind that the dye should be used in a timely manner to reduced dye hydrolysis.

The HC/LC specific protocol does not have a dilution step and uses dye at 200uM in DMSO. Although the protein concentration has been increased, the protein to dye ratio is the same as in the general protocol. This protocol will decrease the reactions a user can perform with one kit because the amount of dye used is higher. However, this protocol should increase the labeling efficiency of the protein.

13. Can I use non-stick eppendorf tubes for the sample preparation procedure?

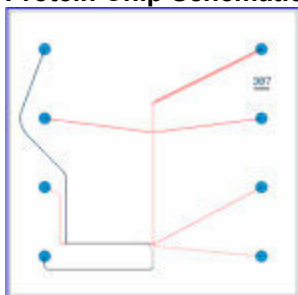
Yes, non-stick eppendorf tubes are preferable when protein samples are at a very low concentration. However, Caliper recommends that users avoid silanized eppendorf tubes which might affect assay performance.

Assay Principles

1. How does the GXII detect protein samples?

The LabChip GX assays are based on traditional gel electrophoresis principles that have been transferred to a chip format. The chip format dramatically reduces separation time and provides automated sizing and quantification information in a digital format. The chip contains an interconnected set of microchannels that join the separation channel and buffer wells. One of the microchannels is connected to a short capillary that extends from the bottom of the chip at a 90-degree angle. The capillary sips sample from the wells of a microplate during the assay. Some of the channels in the chip are larger than others. The larger channels contain buffer. During the chip preparation, the smaller channels and some of the wells are filled with sieving gel and buffer.

Protein Chip Schematic



After the channels are filled, the chip functions as an integrated electrical circuit. The circuit is driven by the 7 electrodes in the electrode cartridge that contact solutions in the chip's wells when the chip holder is closed. Each electrode is connected to an independent power supply that provides maximum control and flexibility. The polymer filling the smaller channels in the chip is designed to sieve proteins by size as they are driven through it by means of electrophoresis, similar to using polyacrylamide gels. In the chip, each sample is sipped by negative pressure until a sufficient quantity is loaded in the chip. The sample is then moved electrophoretically into the central channel. As the fragments move down the central channel, they separate by size, finally passing the laser that excites the fluorescent dye bound to the molecule. The software plots fluorescence intensity versus time and produces an electropherogram for each sample.

2. Can users substitute this dye with another dye?

Caliper does not recommend substitution of the labeling dye with other dyes which may have decreased efficiencies and might be incompatible with its detection optics.

3. How can one improve protein labeling efficiency?

Caliper recommends that users optimize labeling efficiency by evaluating different dye to protein ratios, protein concentration, length of labeling and possibly temperature. Proteins may label differently depending on the number of available lysine residues. Some proteins may only require a short period of time before reaching maximum labeling at a given temperature while others may require longer times, greater amounts of dye or different temperatures. Please see question 2 in protein detection for further discussion.