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Multi-Volume Based Protein Quantification

Methods



Why Quantify Proteins?

Proteins are central to our understanding of biology. In cells, they are multipurpose: from actin providing structural support to proteins acting as enzymes for modulating signal transduction pathways, such as kinases, proteases and phosphatases; to transmembrane proteins that allow for extracellular interactions, such as GPCRs and ion channels. Although almost all proteins are made from the same set of 20 amino acids, their structures and functions are incredibly diverse through the various interactions that can occur through the amino acids that comprise them, their ability to assume quaternary structure and post-translational modifications that can modulate their activity. Many of the studies that probe protein structure and function rely on upstream quantification to ensure suitable results. These downstream studies include mass spectrometry to elucidate primary structure and post-translational modifications, circular dichroism and x-ray crystallography to probe secondary, tertiary and quaternary structure, 2-D gel electrophoresis and western blots to monitor relative protein expression and protein arrays for highly multiplexed protein expression studies.

Fluorescence and absorbance spectroscopy are both common protein quantification methods, although the former is preferred when dilute protein samples are used (i.e. typically <100 µg/mL). In general, absorbance quantification methods provide sufficient analytical performance and are convenient for many studies and laboratories due to the widespread availability of absorbance spectrophotometers. Convenience via increased sample throughput is realized when these methodologies are performed in microplates; and via simplified work flows using small amounts of sample in micro-volume formats. In this application guide, absorbance-based protein quantification methods appropriate for microplate and micro-volume analysis are discussed in detail.

Beer-Lambert Law

Quantification is made possible by the absorption of UV-Vis light by chromophores contained in protein or through the use of colorimetric reagents. The Beer-Lambert Law relates the physical property of light absorption by molecules to concentration within the sample by the following equation:

$$A = \log_{10} \frac{I_0}{I} = \epsilon lc$$

Where A refers to Absorbance, I_0 is the excitation light intensity before passage through the sample, I is the excitation light intensity after passage through the sample, ϵ to the molar absorptivity or extinction coefficient of the analyte, l is the path length (cm) and c is the concentration of the analyte. Spectrophotometers provide the means for the measurement of I_0 and I. Vessels used for holding the sample within the spectrophotometer are typically at a fixed path length, thus if the extinction coefficient of the protein is known, concentrations can simply be determined using the above equation. If the extinction coefficient is not known (i.e. using colorimetric reagents), then calibration curves can be generated such that concentrations can be interpolated.

Instrumentation and Vessels

There are essentially three basic types of instrument used for the spectrophotometric determination of proteins. There are dedicated absorbance spectrophotometers that use cuvettes to hold individual samples, microplate-based spectrophotometers (microplate readers) that use microplates to process many samples rapidly, and dedicated devices for micro-volume analysis. These devices, such as ThermoScientific's NanoDrop series are particularly useful in that only small amounts of protein need be used for quantification. Further, they require no vessel for analysis as samples are directly pipetted onto a pedestal on the instrument. Recently, BioTek Instruments has provided a microplate accessory for use with its microplate readers that allows for micro-volume analysis similar to these dedicated devices. The benefit of the microplate reader and accessory is that many applications can also be performed with the microplate reader (i.e. ELISA). Table 1 provides an overview of the characteristics of vessel types used by these instruments, particularly the number of samples they can process, the path length used for analysis and the sample volume required.

Vessel	Samples	Path length (cm)	Volume (μL)	Vendor
Cuvette	1	1	~3000	Many
	1	1	~1000	
BioCell™	1	1	~1000	BioTek Instruments
Capillary Cuvette	1	~0.1	3	Shimadzu
Microplate	Up to 384	~0.2 to ~1	50 – 200	Many
NanoDrop Pedestal	Up to 8	0.005 – 0.1	0.5 – 2	ThermoScientific
Take3™	Up to 16	0.05	2 – 8	BioTek Instruments
Take3 Trio	Up to 48	0.05	2 – 8	BioTek Instruments

Table 1. Characteristics of vessel types commonly used for protein quantification.

BioTek Instruments offers a range of microplate readers that can not only measure absorbance suitable for protein quantification but also many other detection modes including fluorescence, time resolved fluorescence, fluorescence polarization and luminescence. Each of these microplate readers are capable of multi-volume protein quantification in a wide range of vessels including standard cuvettes (Figure 1), BioTek's patented BioCell™ cuvette which has a fixed 1 cm path length, 96- and 384-well microplates and micro-volume analysis using the Take3™ or Take3™ Trio accessory.

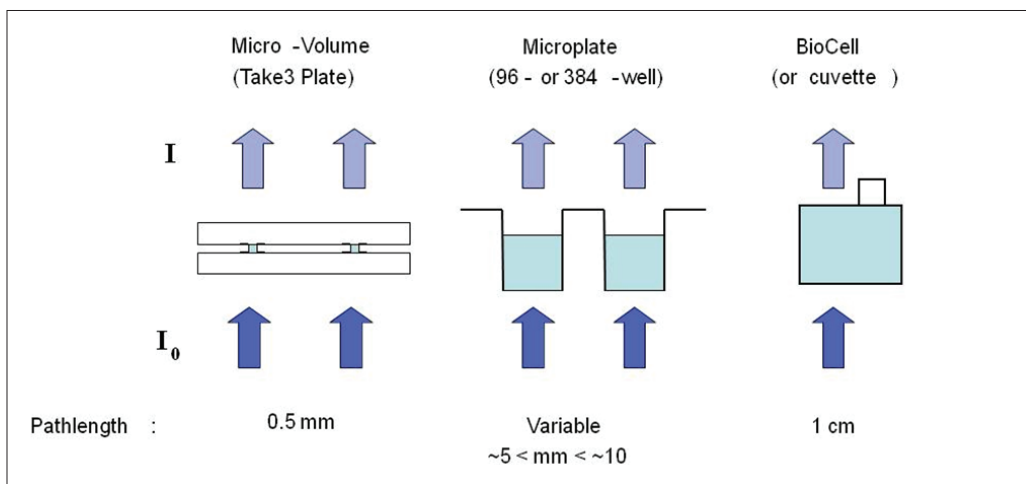


Figure 1. Multi-Volume capabilities of BioTek microplate readers.

Cuvette-based methods are traditional and usually involve sample dilution. While instrumentation is typically inexpensive, simple to operate and provide precise measurements, they offer limited throughput, where measurements are taken on one sample at a time. In some BioTek microplate readers, a dedicated cuvette port is an optional feature, alternatively, either a BioCell or cuvette may be used with the Take3™ accessory.

Microplates are disposable, commonly made of plastics, including low-protein binding versions. Wavelength-specific light from a microplate spectrophotometer moves vertically (Figure 1) through the microplate well and sample. Since the sample volume can vary in microplate format, a path length correction factor must be applied to standardize results to that of a 1 cm path length. BioTek's Gen5™ Data Analysis Software can automatically calculate sample path length by measuring the absorbance peak of water at 977 nm and 900 nm. The difference is then divided by 0.18, which is the absorbance of water at 1 cm, equaling the sample's path length. The step-wise Gen5 procedure is as follows:

BioTek's automated path length correction and quantification (protein example):

1. $A_{977} - A_{900}$ sample OD / 0.18 OD = sample path length (in cm)
2. A_{280} sample - A_{280} blank / sample path length = OD corrected to 1 cm
3. OD corrected to 1 cm * extinction coefficient = protein concentration in the well (in $\mu\text{g/mL}$)

Microplate-based protein quantification offers increased sample throughput and the ability to read higher concentration samples due to the ability to read at shorter path lengths relative to 1 cm cuvette measurements. Additionally, blanks, calibration curves and samples may be prepared and read on the same plate, nearly simultaneously, minimizing incubation time and temperature variations, resulting in increased accuracy.

BioTek's Take3 Micro-Volume Plate (Figure 2) features 16 microspots on custom glass slides suitable for micro-volume analysis. When samples are loaded, the plate forms a fixed 0.5 mm vertical optical path obviating the need for dilution of highly concentrated samples. After the samples are read in BioTek microplate readers, they may be recovered, or the slides wiped clean, as the surface generally resists binding. Note also the ability to perform 1 cm path length determinations with standard cuvettes or BioCells.



Figure 2. Take3 microplate accessory illustrating the 16 microspots, 2 BioCells and a capped standard cuvette.

Protein Quantification Methods

The amino acid residues contained in a protein provide the means for quantification by absorbance spectrophotometry. Some amino acid residues contain natural chromophores, such as the phenyl rings contained in tryptophan, tyrosine and phenylalanine; others provide functional groups that can be targeted with colorimetric reagents. The number of these amino acids vary widely from protein to protein, therefore, the molar absorptivity between proteins may vary greatly. Additionally, sample preparations may contain interfering agents with either similar absorption spectra such as DNA, or detergents and buffers that can affect the derivatization with colorimetric reagents. Each can render sample measurements inaccurate. As a result, the assay format should be chosen based on the desired throughput, available sample quantity, required accuracy and consideration of the presence of potential contaminants. Table 2 includes the most common absorbance-based protein quantification assays in use today.

Protein Functional Group Targeted	Quantification Range (mg/mL for BSA)	Strengths	Issues
Tryptophan, tyrosine, phenylalanine native absorbance	0.2 – 50	No sample preparation; non-destructive; good linear dynamic range	May be considerable error for different proteins; prone to accuracy issues with real samples (i.e. nucleic acid interference)
Peptide bonds, cysteine, tryptophan, tyrosine reduce Cu(II) in Biuret reaction	0.1 – 2	Less variation in results between proteins (more functional groups); compatible with many surfactants used to solubilize proteins	Derivatization chemistry susceptible to interference from copper chelators and reducing agents used to break disulfide bonds
	0.01 – 2	As above, but faster reaction kinetics (20 min @ RT)	
Protein denaturation followed by hydrophobic binding of dye	0.1 – 2	Rapid reaction kinetics compared to BCA	More variation in results between proteins; working reagents susceptible to precipitation by surfactants
	0.01 – 0.1	Even faster reaction kinetics	

Table 2. Common protein quantification methods with typical quantification ranges.

Native Protein Absorbance

The fastest, simplest method to quantify proteins is via ultraviolet (UV) absorbance measurement at 280 nm (A_{280}), whereby light absorbed by the protein directly correlates to protein concentration in accordance with the Beer-Lambert Law. Most proteins have a peak absorbance at this wavelength due to the presence of the aromatic amino acids tryptophan, tyrosine and phenylalanine. A_{280} measurements remain popular, as they are rapid, do not require additional reagents, are non-destructive, and provide good linear dynamic range. Also in some cases, if a sample contains a highly purified protein (i.e. for structural analysis via circular dichroism or x-ray crystallography) and its molar absorptivity is known, concentrations can be determined without the need for calibration curves.

Figure 3 demonstrates the multi-volume capabilities of BioTek's microplate readers. In the figure, comparisons are made between traditional 1 cm path length vessels, such as BioCell to the micro-volume capabilities (0.05 cm) of the Take3 microplate accessory. This was performed with two different instruments: an absorbance microplate reader (Epoch™) and also a multi-mode microplate reader with the capability of detecting multiple detection modes such as fluorescence, luminescence, etc. (Synergy™ 4). The straight line is not a fit to the data point but represents a slope of 1 through the origin. Thus data points that fall on the line demonstrate an equivalence of measurement. Equivalent performance is evident across multi-volumes and BioTek instrumentation.

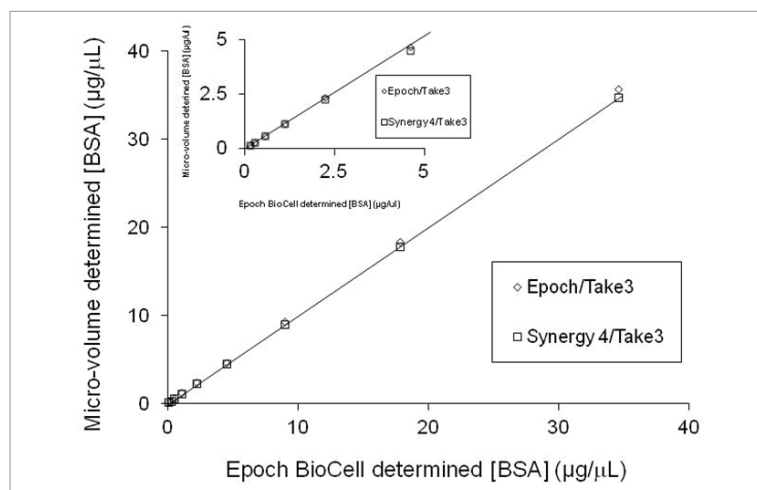


Figure 3. Comparisons between 1 cm path length determinations (BioCell) and micro-volume analysis (Take3) using native protein absorbance at A_{280} . Top left graph illustrates the expanded portion of the larger graph at low BSA concentrations.

In applications that explore protein expression profiling, the sample consists of many different proteins all being expressed at different amounts, each with varying abundance of the phenyl ring-containing amino acids. Therefore molar absorptivities cannot be known. Typically a calibration curve using a model protein, such as bovine serum albumin (BSA) is used to interpolate sample protein mixture concentrations from OD measurements. There will of course be some inaccuracy in this method, the extent determined by the relative expression amounts of each of the proteins and their relative abundance of phenyl ring-containing amino acids compared to BSA. During the protein isolation process, it is also possible to have elution buffers and other sample constituents interfere with the quantification. These sample interferents include nucleic acids, lipids and carbohydrates that can absorb at 280 nm, thus skewing results. Therefore, unless the sample consists of a highly purified individual protein, A_{280} protein determinations are best suited for rapid assessment that provide an estimation of total protein concentration.

Colorimetric Assays

The use of colorimetric reagents tends to provide higher sensitivity determinations relative to A_{280} . Standard protocols typically call for a large excess of working reagent relative to protein to ensure generation of maximum signal from the protein sample. Two of the more common colorimetric reagents are described below.

BCA Assay

The colorimetric bicinchoninic acid (BCA) assay relies on peptide bonds within proteins to reduce Cu^{2+} to Cu^+ under alkaline conditions. The Cu^+ ions then chelate with two BCA molecules, producing a purple complex that strongly absorbs at 562 nm. The BCA assay uses the same principle as the older Lowry assay, with modifications that result in formation of a more stable detection reagent in alkaline conditions and requiring only a single step process. BCA assays are less affected by protein structural variations than UV spectroscopic methods, although signal generation is proportional to the number of peptide bonds in the protein exposed to the aqueous environment. The reagent is compatible with many surfactants used for protein solubilization.

The BCA assay requires a standard curve (Figure 4) be run simultaneously with sample, and a necessary incubation step. The BCA assay is incompatible with copper chelators, buffers with high buffering capacity and reducing agents. The higher sensitivity of the BCA assay relative to native protein absorbance at A_{280} is evident in Figure 4.

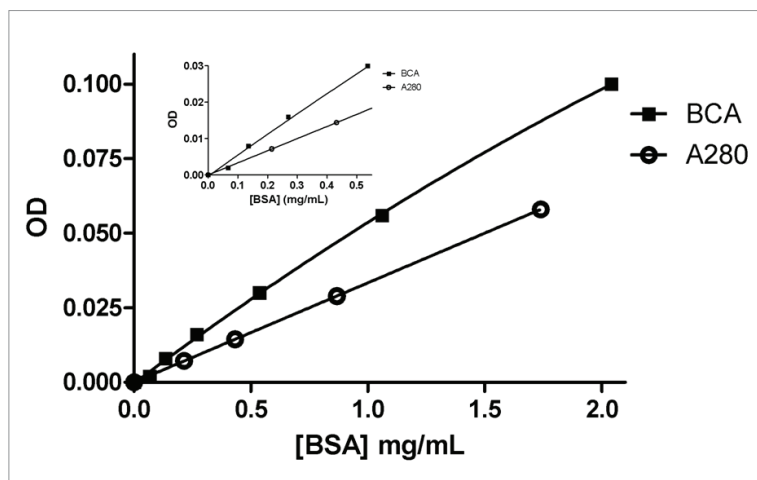


Figure 4. Calibration curves for the protein bovine serum albumin (BSA) using both native absorbance at A_{280} and with the colorimetric reagent BCA. Micro-volume analysis with 2 mL samples on the Take3 microplate accessory with BioTek's Epoch microplate reader.

Bradford Assay

The colorimetric Bradford assay can be used to rapidly measure protein concentration by use of Coomassie Brilliant Blue G-250 dye. In acidic solutions, the dye is in a negatively charged red form that donates electrons to the protein's ionizable groups resulting in partial denaturation of the protein. The hydrophobic portion of the dye then binds to exposed hydrophobic pockets, and the negatively charged region creates ionic bonds with nearby positively charged amine groups of the protein. This results in a visible shift to a stable blue form with an absorbance spectrum maximum at 595 nm indicative of protein levels in solution.

The Bradford assay is faster and more stable than the aforementioned BCA assay, however, the response will vary with protein composition and is susceptible to interference, and possible formation of precipitate, by elevated detergent and buffer concentrations. The assay requires use of a standard curve, run at the same time as the sample, and may require sample dilution to fit within the linear range of the assay due to nonlinearity at very high protein concentrations.

Micro-Volume *In-Situ* BCA Analysis

Micro-volume methods, using a modified protocol that calls for changes in the relative concentrations of protein and BCA working reagent from a 20:1 volume ratio of BCA working reagent to protein to a 1:1 ratio, can shift the working range of the BCA calibration curve relative to microplate- or cuvette-based measurements. The Take3 plate is particularly suited for this modified protocol as sample and working reagent can be sequentially added directly to the microspots of the Take3 plate and the reaction incubation and subsequent analysis performed therein. This “in-situ” assay workflow (Figure 5) is simple and resembles the workflow for a typical microplate assay where reagents and samples are added sequentially to microplate wells without the need for offline color development. This significantly improves ease of use. Further, as only 2 μ L of sample is consumed, this promotes sample conservation.

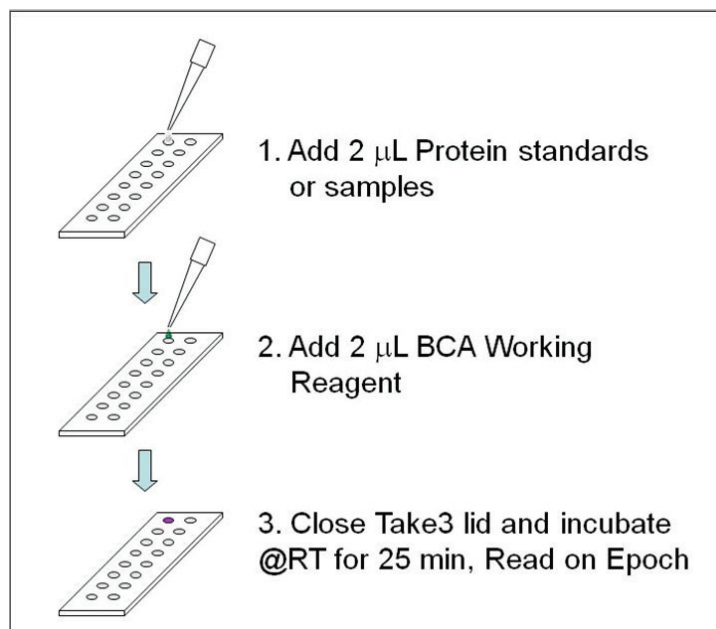


Figure 5. *In-situ* work flow for micro-volume BCA analysis using a 1:1 BCA working reagent to sample ratio with Take3 accessory. Total assay volume is 4 μ L.

The *in-situ* work flow provides two main advantages over the standard 20:1 protocols. First, the assay volume is only 4 μL , which leads to fast reaction kinetics. The reaction is complete in less than 30 minutes compared to the typically recommended 2 hours in the 20:1 protocol. Also, the sensitivity of the assay is improved by the significant reduction in protein dilution with the BCA reagent, which extends the dynamic range to much lower protein concentrations to a limit of quantification of about 0.01 mg/mL BSA (Figure 6).

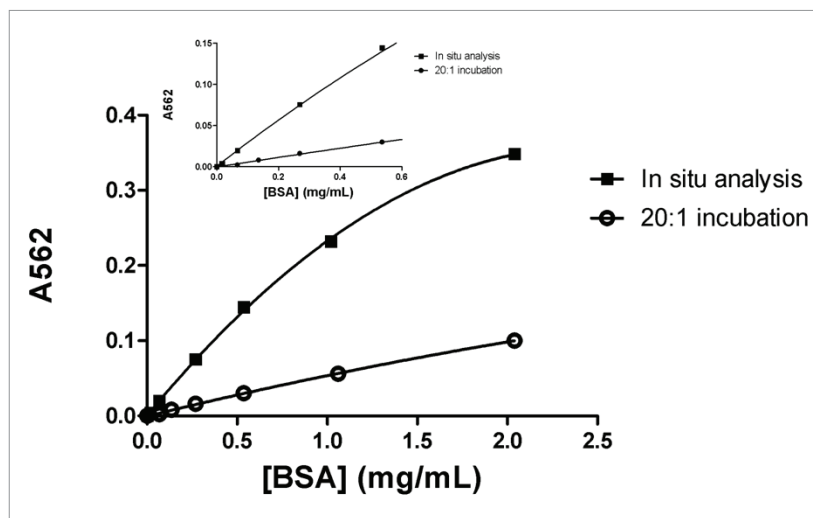


Figure 6. Take3 micro-volume analysis comparison between an *in situ* assay and one conducted in a micro-centrifuge tube using a 20:1 volume ratio of BCA working reagent to BSA standard.

Protein Purity Assessment

In addition to protein quantification, an assessment of protein purity is essential for downstream applications. The A_{260}/A_{280} ratio, where samples are measured at the nucleic acid absorbance peak (A_{260}) and again at the protein absorbance peak (A_{280}), is commonly used to approximate nucleic acid purity. This measurement may also be used to estimate protein purity where a ratio of 0.60 is generally indicative of a pure protein sample.

As seen in Table 3, very low levels of nucleic acid contamination can have a large effect on the A_{260}/A_{280} ratio when estimating protein purity. Proteins are generally considered pure if results indicate 95-99% purity, depending on the downstream application. Confirmation of protein purity can be achieved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis or the more sensitive silver staining method can be used to determine the purity of proteins present at very low concentrations.

% DNA	% Protein	% Accuracy [DNA]	% Accuracy [Protein]
100.0	0.0	0.0	N/A
59.7	40.3	9.6	1107.3
39.7	60.3	18.5	445.9
27.8	72.2	29.0	285.3
19.8	80.2	44.3	189.0
14.1	85.9	62.9	121.4
9.9	90.1	91.5	86.1
6.6	93.4	139.2	57.4
4.0	96.0	226.8	28.5
1.8	98.2	498.0	15.7
0.0	100.0	N/A	0.0

Table 3. Calculated percent accuracy determinations from spectral data of varying DNA/protein mixtures. Extinction coefficients of 50 ng/ $\mu\text{L}/\text{OD}$ and 0.667 $\mu\text{g}/\mu\text{L}/\text{OD}$ for DNA and protein, respectively, were used to compare calculated determinations versus expected values, expressed as percent accuracy.

Best Practices

- The assay method should be best suited to the sample type and conditions.
- Use appropriate precautions to protect samples from foreign debris and contamination.
- Replicate sample testing improves result confidence.
- Colorimetric protein assays require standard curves each time the assay is performed.
- When working in microplate formats, choose flat-bottomed and optically clear plates.

Protein Quantification via BioTek's Microplate Instruments

BioTek has a number of microplate-based instruments for protein measurement via absorbance and fluorescence.

BioTek Microplate Reader	Absorbance	Fluorescence	Gen5™ Software	Take3™ Micro-Volume Plate Compatible
Synergy™ NEO Hybrid Multi-Mode Microplate Reader	•	•	•	•
Synergy H4 Hybrid Multi-Mode Microplate Reader	•	•	•	•
Synergy H1 Hybrid Multi-Mode Microplate Reader	•	•	•	•
Synergy Mx Monochromator-Based Multi-Mode Microplate Reader	•	•	•	•
Synergy 2 Multi-Mode Microplate Reader	•	•	•	•
Synergy HT Multi-Mode Microplate Reader	•	•	•	•
PowerWave™ Microplate Spectrophotometer	•			
Eon™ Microplate Spectrophotometer	•			•
Epoch™ Microplate Spectrophotometer	•			•
ELx808™ Absorbance Microplate Reader	•			
ELx800™ Absorbance Microplate Reader	•			
FLx800™ Fluorescence Microplate Reader		•		

For more information on protein quantification methods, low volume measurements or BioTek's microplate readers, contact your local representative or CustomerCare@BioTek.com.

DE44321.2042824074

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August 1, 2021