Isotope Dilution Mass Spectrometry is an accurate method to determine hemagglutinin concentration of influenza virus standards

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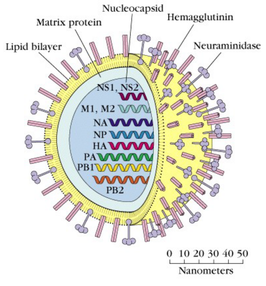
Abstract

Hemagglutinin (HA) is currently used as the marker for influenza vaccine potency. The amount of HA is measured using a single radial immunodiffusion assay (SRID) that provides an indirect measurement of antigenically intact HA relative to a reference that is calibrated against a primary liquid standard (PLS). The HA concentration of PLS is traditionally determined from densitometry of SDS-PAGE bands and total protein concentration. More advanced methods are now available to measure protein concentration, including mass spectrometry (MS)-based methods. In this report, we use an isotope dilution mass spectrometry (IDMS) method to quantify the HA in PLSs. IDMS combines the sensitivity and selectivity of mass spectrometry (MS) with the precision and accuracy associated with the use of isotopically labeled internal standards. We optimized the IDMS method by treating samples with detergent prior to digestion with trypsin. HA concentrations of PLSs prepared using egg and cell production platforms were measured by IDMS and SDS-PAGE methods. The data showed excellent reproducibility of the IDMS method and comparable concentrations measured by IDMS and SDS-PAGE methods for egg PLSs, but not for cell grown PLSs. Intact mass analysis indicated the presence of host cell proteins in cell grown PLSs which were not taken into account during SDS-PAGE analysis. Electron microscopy data further confirmed the differences in quality of PLS from different sources. In conclusion, IDMS is suitable for accurate determination of HA concentration in PLSs from all platforms. This advanced technique expedites the calibration of reference standards for influenza vaccines and ensures potency is correctly measured.

Introduction

Influenza (flu) is a highly contagious respiratory illness caused by influenza viruses. An annual seasonal influenza vaccine is the best way to help protect against flu. The effectiveness of current licensed influenza vaccines is dependent on the amount of HA from each component strain, which is measured by SRID using reference reagents calibrated against a primary liquid standard (PLS). Accurate determination of the amount of HA in the PLS is therefore critical. This has traditionally been calculated from %HA determined by SDS-PAGE and total protein measured by Lowry assay. Co-migration of other proteins with HA, diffuse glycosylated protein bands, and protein impurities can lead to incorrect determination of %HA by SDS-PAGE, especially in cell grown PLSs. An isotope dilution mass spectrometry (IDMS) method has been evaluated as an alternate physicoehcmical method to measure HA content in the PLS.



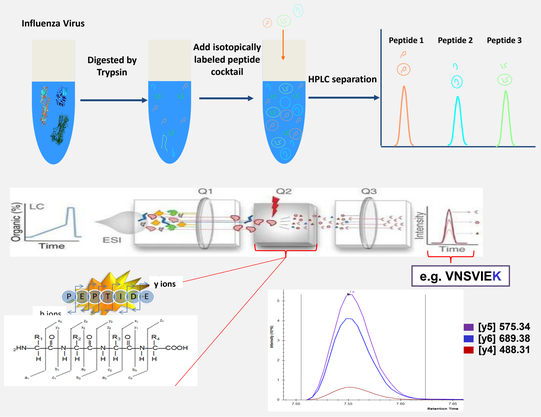
Influenza virus

- An enveloped virus with 8 negative single stranded RNA segmented genome.
- Roughly spherical particles, ranging from 80 to 120 nm in diameter.
- Hemagglutinin (HA) and neuraminidase (NA) are glycoproteins on the outside of the viral particles. The potency of inactivated influenza vaccines is based on HA content.

Materials and Methods

Isotope-dilution Mass Spectrometry (IDMS)

IDMS combines the sensitivity and selectivity of mass spectrometry (MS) instrument with the precision and accuracy associated with the use of isotopically labeled compound (internal standard) to overcome the shortcoming of SDS-PAGE. To increase selectivity and sensitivity, high-performance liquid chromatography (HPLC) and multiple reaction monitoring (MRM)/selected reaction monitoring (SRM) assay are applied by IDMS.



HA quantitation by IDMS

- Specific target peptide(s) are selected based on MS response and location in full length protein sequence. Peptides selected for quantitation of HA in H1N1 A/Victoria HA are shown below as an example.

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MKAILVVMLY TFTTANADTL CIGYHANNST DTVDTVLEKN VTVTHSVNLL EDKHNGKLCK
LRGVAPLHLG QCNIAGWILG NPECESLSTA RSWSYIVETS NSDNGTCYPG DFINYEELRE
QLSSVSSFER FEIFPKTSSW PNHDSDNGVT AACSHAGAKS FYKNLIWLVK KGKSYPKINQ
TYINDKGKEV LVLWGIHHPP TITDQESLYQ NADAYVFVGT SRYSKKFKPE IAARPKVRDR
AGRMNYYWTL VEPGDKITFE ATGNLVAPRY AFTMEKEAGS GIIISDTPVH DCNATCQTPE
GAINTSLPFQ NVHPITIGKC PKYVRSTKLR LATGLRNVPS IQSRGLFGAI AGFIEGGWTG
MVDGWYGYHH QNDQGSGYAA DLKSTQNAID KITNKVNSVI EKMNTQFTAV GKEFNHLEKR
IENLNKKVDD GFLDVWTYNA ELLVLLENER TLDYHDSNVK NLYEKVRHQL KNNAKEIGNG
CFEFYHKCDN TCMESVKNGT YDYPKYSEEA KLNREKIDGV KLDSTRIYQI LAIYSTVASS
LVLVVSLGAI SFWMCSNGSL QCRICI
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- PLS and QC samples are denatured and digested with trypsin after Zwittergent and Rapigest treatment.
- A known amount of synthetic reference peptide, in which one amino acid has been isotopically labeled, is spiked into the sample.
- Standards and samples are evaluated by HPLC-mass spectrometry.
- The ratio of the peak area of the isotopically labeled reference peptide and the endogenous target peptide is used to calculated the molar amount of HA.
- HA concentration is calculated based on the MW of protein sequence.

HA quantitation by SDS-PAGE/total protein

- HA bands are identified by comparing bands of non-reduced/reduced SDS-PAGE analysis.
- Preparation is purified virus, therefore density of HA band is measured in proportion to other viral bands.
- HA concentration= %HA x total protein concentration.

Results and Discussion

Table 1. Zwittergent increases PLS solubility and digestion efficiency. The table below shows protein and IDMS results of A/Singapore/GP1908/2015 IVR-180 PLS in ammonium bicarbonate (ABC), and 2 detergents (Rapigest and Rapigest+ zwittergent.

Protein in supernatant of samples centrifuged after detergent addition	
Diluent	**Total protein concentration (µg/mL)**
50mM ABC	1081.95
0.2% Rapigest	1201.79
0.5% Zwittergent + 0.2% Rapigest	1383.76
HA (µg/mL): IDMS average of replicates(3 peptide/3 replicates)	
Diluent	**HA concentration (µg/mL)**
50mM ABC	146.87
0.2% Rapigest	159.47
0.5% Zwittergent+ 0.2% Rapigest	192.29

Table 2. HA concentrations measured by IDMS and SDS-PAGE are similar for PLS grown in eggs but not in cells.

Virus Strains	Strain Type	IDMS (µg/mL)	SDS-PAGE (µg/mL)	IDMS/ SDS-PAGE
Egg Grown PLS Samples				
A/Victoria/2570/2019 (IVR-215) PLS Lot# 2020/134B	H1N1	185.79	182.77	1.02
A/Brisbane/2/2018 (IVR-190) Lot# 2019/127B	H1N1	140.80	152.41	0.92
IRC-0036 A/Switzerland/8060/2017 NIB-112 NIBSC PLS-B	H3N2	184.11	164.05	1.12
A/Darwin/9/2021 SAN-010 PLS Lot#U00026341	H3N2	245.57	248.47	0.99
B/Michigan/01/2021 (wt) PLS Lot#U00026339	B	640.80	537.30	1.19
B/Colorado/6/2017 Lot: 2018/124B	B	142.15	150.37	0.95
A/Astrakhan/3212/2020 (IDCDC-RG71A) PLS Lot#AFPNAUA003	H5N8	226.08	256.94	0.88
A/Guangdong/17SF003/2016 NIBRG-375 PLS Lot# IRC-0039	H7N9	136.07	155.65	0.87
Cell Grown PLS Samples				
A/Singapore /TT1384/2016 PLS #257809	H1N1	214.67	461.15	0.47
A/Idaho/07/2018 PLS (cell) Lot #258684	H1N1	253.08	558.87	0.45
A/Darwin/11/2021 PLS Lot#326768	H3N2	396.42	544.85	0.73
A/Indiana/08/2018 PLS Lot#259225	H3N2	337.15	634.05	0.53
B/HongKong/259/2010	B	524.53	971.60	0.54
B/Iowa/06/2016 PLS #200464	B	328.73	675.84	0.49
A/Astrakhan/3212/2020 PLS Lot# 354398	H5N8	272.44	600.60	0.45
A/Guangdong/17SF003/2016 (H7N9) Lot#254521	H7N9	113.52	473.61	0.24

Figure 1. Egg-grown viruses have expected electron microscopic images whereas viruses cultured in cells are often not uniform.

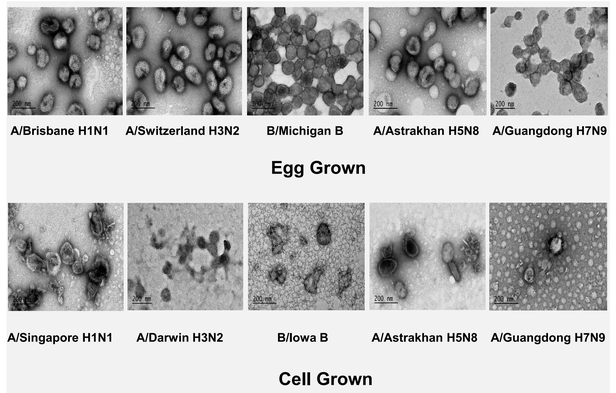
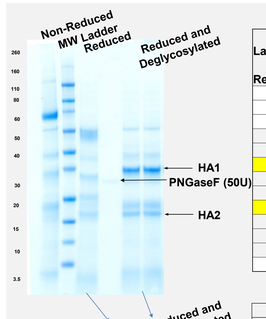


Figure 2. Cell cultured PLS contains a large number of impurities that contribute to inaccurate SDS-PAGE measurements.



Lane 5	B/Hong Kong/259/2010 (cell based)			2016-06-21-A 5 ug 4-12%
Region	Approx MW	net intensity	% intensity	gel
1	257.40	1012.35	0.52	
2	115.90	4415.74	2.26	
3	61.90	11415.06	5.85	
4	53.91	5509.72	2.82	
5	48.06	17839.57	9.14	
6	40.00	47492.95	24.32	HA1
7	36.46	14455.48	7.40	
8	27.29	24682.45	12.64	
9	23.75	23668.7	12.12	HA2
10	20.42	4758.58	2.44	
11	14.69	11506.34	5.89	
12	8.13	28527.56	14.61	
			%HA	36.44

hemagglutinin	B/HongKong/259/2010
matrix protein 1	
nucleoprotein	
proteasome subunit beta	Canis Lupus Failimaris
annexin A1	
annexin A2	
proteasome subunit alpha	
polyubiquitin-C	
proteasome subunit beta	
junction plakoglobin	
glyceraldehyde-3-phosphate dehydrogenase-like	
peroxiredoxin-1	
actin_ cytoplasmic 2	
glyceraldehyde-3-phosphate dehydrogenase	
rho GTPase-activating protein	
proteasome subunit alpha	
peroxiredoxin-2	
ADP/ATP translocase 2	
myomesin-2	
E3 ubiquitin-protein ligase TRAIP	
glial fibrillary acidic protein	

Conclusion

- **IDMS is an accurate, quick, high precision targeted method that provides accurate measurement of HA in both egg and cell grown PLSs.**
- **IDMS overcomes the shortcoming of SDS-PAGE in the determination of HA concentration in PLS and thereby ensures flu vaccine HA content.**

Acknowledgement

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