Title

Protocol for preparing murine tissue for comparative proteomics study of vaccine adjuvant mechanisms.

Journal

STAR Protocols, 4(3)

Authors

Li, Yibo

Chen, Xinyuan

Publication Date

2023-09-15

DOI

10.1016/j.xpro.2023.102396

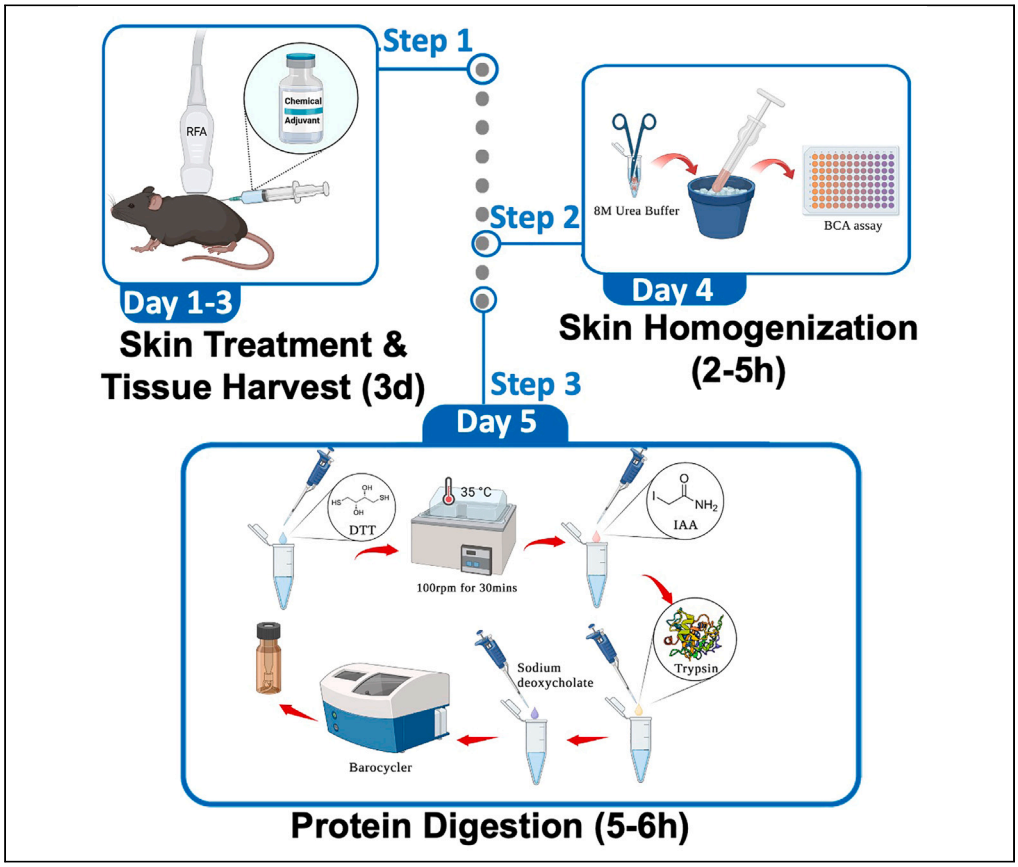
Peer reviewed

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for preparing murine tissue for comparative proteomics study of vaccine adjuvant mechanisms



Understanding the mechanisms of action of adjuvants at the tissue level is crucial to the development of more potent and safer versions for human use. Comparative tissue proteomics presents a novel tool to study their unique action mechanisms. Here, we present a protocol for preparing murine tissue for comparative proteomics study of vaccine adjuvant mechanisms. We describe steps for adjuvant treatment in live animals, tissue harvesting, and homogenization. We then detail protein extraction and digestion to prepare for liquid chromatography-tandem mass spectrometry analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Yibo Li, Xinyuan Chen

xchen14@uri.edu

Highlights

Different adjuvants induce distinct tissue proteome changes

Identify proteins for each adjuvant by comparative tissue proteomics analysis

Screen proteins to study adjuvant mechanisms with traditional approaches

Comparative tissue proteomics enables study of vaccine adjuvant mechanisms

Li & Chen, STAR Protocols 4, 102396
September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102396

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for preparing murine tissue for comparative proteomics study of vaccine adjuvant mechanisms

Yibo Li[1] and Xinyuan Chen[1,2,3,*]

[1]Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, 7 Greenhouse Road, Avedisian Hall, Room 480, Kingston, RI 02881, USA

[2]Technical contact

[3]Lead contact

*Correspondence: xchen14@uri.edu
https://doi.org/10.1016/j.xpro.2023.102396

SUMMARY

Understanding the mechanisms of action of adjuvants at the tissue level is crucial to the development of more potent and safer versions for human use. Comparative tissue proteomics presents a novel tool to study their unique action mechanisms. Here, we present a protocol for preparing murine tissue for comparative proteomics study of vaccine adjuvant mechanisms. We describe steps for adjuvant treatment in live animals, tissue harvesting, and homogenization. We then detail protein extraction and digestion to prepare for liquid chromatography-tandem mass spectrometry analysis.

For complete details on the use and execution of this protocol, please refer to Li et al.[1]

BEFORE YOU BEGIN

This protocol describes *in vivo* animal treatment with our recently developed physical radiofrequency adjuvant (RFA)[1–5] and traditional chemical adjuvants,[6–8] skin collection, and downstream processing to prepare for proteomics LC-MS/MS analysis. The lateral back skin of mice is exposed to the physical RFA treatment or intradermally injected with chemical adjuvants, such as MF59-like AddaVax, monophosphoryl lipid A (MPL), Alum, and MPL/Alum mixture. The chemical adjuvants are licensed for delivery into muscles.[9,10] The reason to choose intradermal delivery in this protocol is because RFA acts on skin to boost intradermal vaccination.[1–5] The delivery of chemical adjuvants into the skin allows the direct comparison of physical and chemical adjuvants-induced tissue proteome changes. However, the procedures described in this protocol can be readily adapted to extract proteins from other tissues (e.g., muscle, draining lymph nodes) or cultured cells (e.g., dendritic cells, peripheral blood mononuclear cells).

The first part of the protocol describes mainly animal procedures that include chemical and physical adjuvant treatment and skin harvesting.

The second part of the protocol describes *in vitro* procedures related to protein extraction, quantification, denaturation, trypsin-digestion, and various other steps to prepare samples for LC-MS/MS analysis.

Institutional permissions

C57BL/6 mice were purchased from Jackson Laboratories. All animal procedures were approved by the Institutional Animal Use and Care Committee (IACUC) of University of Rhode Island with approval number AN1516-004. Please ensure to acquire permission from your institutions for

animal experiments before the experiment. Make sure your animal procedures will be performed in accordance with Institutional and National guidelines and regulations.

Adjuvant preparation

Timing: around 20 min

1. Determine the types of adjuvants to be included in the study: AddaVax, MPL, Alum, MPL/Alum, and RFA.
2. AddaVax preparation: AddaVax, an oil-in-water nano-emulsion containing squalene, is formulated similarly to MF59 (a licensed adjuvant for influenza vaccine). In this study, it is prepared by mixing with phosphate-buffered saline (PBS) at 1:1 volume/volume ratio for injection.
3. MPL preparation: MPL (1 mg) in the powder form will be first dissolved with 100 μL DMSO and then 300 μL water. After that, MPL will be mixed with PBS at 1:1 volume/volume ratio.
4. Alum preparation: Alum adjuvant (Alhydrogel, 2%) will be mixed with PBS at 4:1 volume/volume ratio.
5. MPL/Alum preparation: the above prepared MPL adjuvant is mixed with Alum (Alhydrogel, 2%) at 1:1 volume/volume ratio.
6. The physical RFA device is fully charged. In this study, a cosmetic fractional bipolar RF device equipped with 12 × 12 array of microelectrodes in 2 × 2 cm^2 area is used. The red light in the front will turn green when fully charged.

⚠ CRITICAL: It's recommended to include at least 3–4 different adjuvants to identify uniquely induced proteins by each adjuvant to facilitate the downstream mechanism studies. Freshly prepare the adjuvants based on the manufacturer's recommendations.

Animal experiment preparation

Timing: 1–2 months

7. Get animal protocol approved by the Institutional Animal Use and Care Committee (IACUC).
8. Order animals (at least 5 mice/group) and allow the required acclimation time (usually 1 week).
9. Autoclave scissors and forceps for skin collection.

Urea buffer preparation

Timing: 10 min

We prepare a final volume of 25 mL 8 M urea buffer.

10. Dissolve 12.01 g Urea (60.06 g/mol) in 16 mL deionized water.
11. After complete dissolution, make up volume to 25 mL.

Note: Urea dissolution is an endothermic process. A hotplate stirrer can be used to speed up the dissolution process. The urea buffer can be stored at around 25°C for up to 1 year.

Dithiothreitol (DTT) stock solution preparation

Timing: 30 min

We prepare a final volume of 10 mL 1 M DTT stock solution.

12. Dissolve 1.54 g DTT (154.25 g/mol) in 10 mL deionized water.

13. After complete dissolution, filter through 0.22 μm syringe filter.
14. Aliquot 1 mL per tube and store at −20°C for a maximum of 6 months.

⚠ CRITICAL: Wear protective gloves, protective clothing and protective eyeglasses when handling DTT because it is harmful if swallowed and irritating to eyes and skin.

Alternatives: DTT of 100 mM can also be freshly made for use in this protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
AddaVax™	InvivoGen	Cat# vac-adx-10
Alhydrogel	InvivoGen	Cat# vac-alu-250
Lipid A, monophosphoryl (MPL)	Sigma	Cat# L6895
Dithiothreitol (DTT)	Thermo Fisher Scientific	Cat# R0862
Iodoacetamide (IAA)	Sigma	Cat# I1149
Ammonium bicarbonate	Sigma	Cat# 09830
Sodium deoxycholate	Sigma	Cat# 30970
TPCK-treated trypsin	Sigma	Cat# 4370285
Gibco™ PBS, pH 7.4	Thermo Fisher Scientific	Cat# 10010023
Methanol	Sigma	Cat# 179337
Chloroform	Sigma	Cat# 2432
Formic acid	Fisher Scientific	Cat# A117-50
LC-MS grade acetonitrile	Millipore Sigma	Cat# 1.00029
LC-MS grade water	Fisher	Cat# W6-4
Urea	Fisher	Cat# U15-3
Bovine serum albumin (BSA)	Sigma	Cat# A2153
Critical commercial assays		
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23227
Hair Nair (hair removal lotion)	Nair	N/A
Experimental models: Organisms/strains		
C57BL/6 mouse (male, 6 weeks old)	Charles River Laboratories	N/A
Other		
Digestion tubes (PCT MicroTubes)	Pressure Biosciences Inc.	Cat# MT-96
MicroCaps	Pressure Biosciences Inc.	Cat# MC150-96
RF device	Norlanya Technology Co.	N/A
Microplate reader (SpectraMax iD3)	Molecular Devices	N/A
Microplate reader software (SoftMax Pro 7.1)	Molecular Devices	N/A
Barocycler NEP2320	Pressure Biosciences Inc.	N/A
Sciex 5600 TripleTOF™ mass spectrometer	Sciex	N/A
2 mL glass grinder	Wheaton	N/A
Shaking water bath	Thermo Fisher	Model # FSSWB15

MATERIALS AND EQUIPMENT

Methanol:chloroform:water mixture (2:1:1, v/v/v)		
Reagent	Final concentration	Amount
Methanol	50%	5.0 mL
Chloroform	25%	2.5 mL
Water (diH_2O)	25%	2.5 mL
Total		**10 mL**
Store at 4°C for up to 1 year. This recipe is for 10 samples.		

50 mM Ammonium bicarbonate (pH 8.0) + 3% Sodium deoxycholate (DOC)		
Reagent	Final concentration	Amount
Ammonium bicarbonate (MW = 79.06 g/mol)	50 mM	59.3 mg
Sodium deoxycholate	3%	0.45 g
Water (diH_2O)	N/A	15 mL
Total		15 mL
Prepare freshly for the same-day use. This recipe is for >100 samples.		

5% Formic acid		
Reagent	Final concentration	Amount
Formic acid	5%	150 μL
LC-MS grade acetonitrile	47.5%	1425 μL
LC-MS grade H_2O	47.5%	1425 μL
Total		3 mL
Store at 4°C for up to 1 year. This recipe is for 20 samples.		

- 200 mM Iodoacetamide (IAA) solution: Dissolve 36.98 mg IAA (184.96 g/mol) in 1 mL deionized water.

  ⚠ CRITICAL: IAA has acute toxicity and respiratory sensitization. Use IAA only in a chemical fume hood and immediately change contaminated clothing. Always use the equipment for eye/face protection and wear protective gloves and clothing when using IAA.

  Note: IAA is light sensitive. Wrap IAA tube with aluminum foil to prevent light exposure. Always prepare fresh IAA solution for use in this protocol.

- Ice-cold Methanol: Place pure methanol on ice or at −20°C for at least 30 min before use.

  ⚠ CRITICAL: Methanol is highly flammable and toxic. Use methanol in a chemical fume hood and use the equipment for eye/face protection and wear protective gloves and clothing when handling methanol.

  Note: The ice-cold methanol can be stored at −20°C for up to 1 year.

- TPCK-treated trypsin: Reconstitute 0.5 mg trypsin powder with 0.5 mL deionized water to make 1 mg/mL stock solution.

  Note: Keep at 4°C for same day use and can be stored at −20°C for up to 1 year.

STEP-BY-STEP METHOD DETAILS

Skin treatment and tissue harvest

Timing: 3 days

Hair of the lateral back skin of mice is removed the day before experiment. Hair-removed skin is intradermally injected with diverse types of chemical adjuvants or treated with physical RFA. Skin is collected 18 h later.

1. Day 1 Hair removal.
   a. Anesthetize mice as approved by your IACUC protocol.
   b. Remove hair on lateral dorsal skin of mice with scissors to create an area of more than 2 × 2 cm^2, and then carefully apply hair Nair for 2 min followed by gently rinsing with warm tap water.

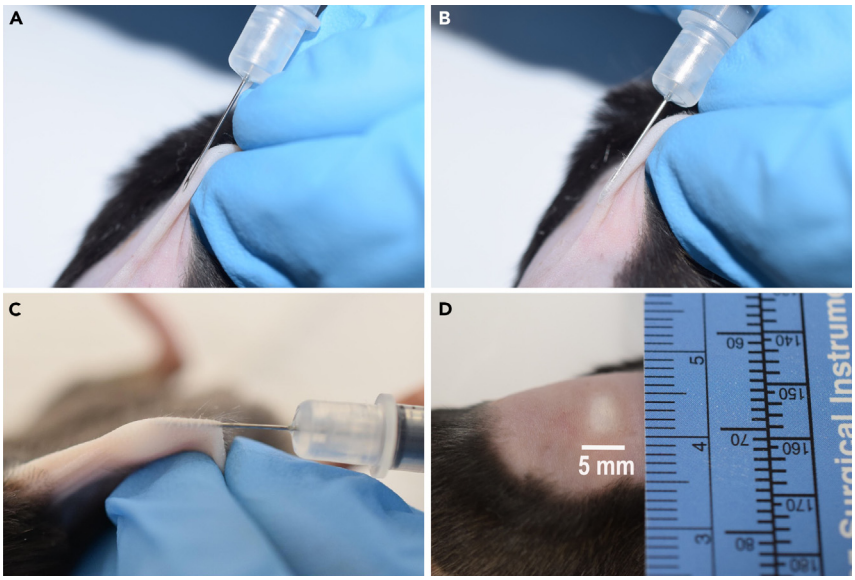


Figure 1. Intradermal injection of chemical adjuvants

(A) Place the needle, bevel up, on the skin.

(B) Gently insert the needle into the dermal layer of the skin.

(C) Slowly advance by ~5 mm and inject 20 μL chemical adjuvants.

(D) A small bleb forms in the skin.

c. Keep mice on 37°C warming pad for recovery.

Note: Step 1 takes about 1–2 h.

2. Day 2 RFA treatment.
   a. Anesthetize mice appropriately.
   b. Apply a thin layer of ultrasound coupling medium on the hair-free skin of mice.
   c. Activate RFA device and set the energy level at high.
   d. Firmly press the front tip of the RFA device on the coupling medium-applied skin and hold for 90–120 s for RFA treatment.
   e. Dry RFA-treated skin with Kimwipe and mark the treatment area with a sharpie marker.
   f. Intradermally inject 20 μL sterile PBS to the RFA treated skin using the Mantoux method,[11] as shown in Figure 1.

Note: The red indicator light should be always on during the treatment. This step takes about 1 h.

3. Day 2 Sham treatment.
   a. Anesthetize mice appropriately.
   b. Apply a thin layer of ultrasound coupling medium on the hair-free skin of mice.
   c. Firmly press the front tip of the RF device with the power off on the coupling medium-applied skin and hold for the same period of time.
   d. Dry RFA-treated skin with Kimwipe and mark the treatment area with a sharpie marker.
   e. Intradermally inject 20 μL sterile PBS to the Sham-treated skin as above.

Note: Make sure the power of the RF device is off. This step takes about 1 h.

4. Day 2 Chemical adjuvant treatment.
   a. Anesthetize mice appropriately.

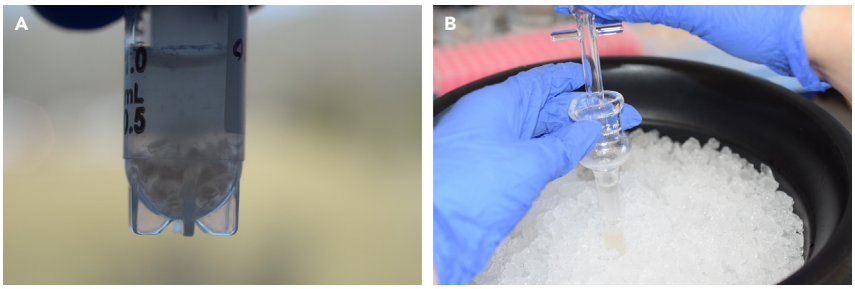


Figure 2. Tissue Homogenization
(A) Cut tissue into small pieces with scissors.
(B) homogenize tissue using a glass grinder on ice.

b. Intradermally inject 20 μL chemical adjuvants to the same anatomical location of the skin as above.
c. Mark the chemical adjuvant-treated area with a sharpie marker.

Note: This step takes about 1 h.

5. Day 3 Skin collection.
   a. Euthanize mice with appropriate methods.
   b. Collect center regions of RFA-treated skin and entire chemical adjuvant-treated skin according to the Sharpie marker labels 18 h after treatment.
   c. Weigh skin weight and then freeze skin in dry ice or liquid nitrogen.

Note: Remove the fat tissue using forceps and scissors while harvesting the skin. This step takes about 1 h.

Pause point: Skin tissue can be stored at −80°C for several months.

Protein extraction

Timing: 2–5 h

Skin tissue is cut into small pieces followed by homogenization in 8 M Urea buffer. Skin homogenates are then centrifuged. Supernatants are collected for downstream processing.

6. Sample preparation.
   a. Transfer skin tissue from −80°C to an Eppendorf tube.
   b. Add 1 mL 8 M Urea buffer to the tube.
   c. Cut the skin tissue into small pieces of less than 2 mm in any dimensions (Figure 2A) with scissors.

Note: Keep the tubes on ice while cutting the tissue. The cutting step facilitates the release of most of the tissue proteins during the subsequent homogenization step. We used the same volume of Urea buffer since we believe this volume is sufficient to extract total proteins from the biggest skin tissues collected according to the tissue weight in Table 1. Another strategy is to add 8 M Urea proportionally to skin weight.

7. Homogenization.
   a. Transfer the tissue with the 8 M urea buffer into a 2 mL glass grinder.
   b. Thoroughly homogenize the skin tissue with the glass grinder on ice as shown in Figure 2B.

Table 1. Skin weight and protein concentration

Groups	Sample #	Skin weight (mg)	Protein concentration (μg/mL)
Sham	1	35.0	1494.49
	2	24.2	900.28
	3	67.7	1711.27
RFA	4	56.5	3420.8
	5	42.0	1038.85
	6	61.4	1928.04
AddaVax	7	82.2	4603.27
	8	40.9	2020.7
	9	50.8	1798.83
Alum	10	26.7	682.66
	11	31.6	806.77
	12	72.3	2288.48
MPL	13	29.0	1429.04
	14	38.3	2124.41
	15	52.5	1593.11
MPL/Alum	16	44.1	671.61
	17	62.5	3371.49
	18	57.6	2886.09

Note: Keep the tissue cold while homogenizing. Skin tissue is 'hard' to homogenize due to its high contents of collagen.

c. Transfer homogenized tissue with the buffer to new Eppendorf tubes including the remaining 'hard' skin tissue that cannot be homogenized.
d. Centrifuge at 16,000 × *g* for 10 min at 4°C.
e. Collect supernatants into new Eppendorf tubes.

Note: Collect only the supernatant. Centrifuge again to obtain pure supernatant if needed.

Protein concentration quantification

Timing: 1–2 h

The protein concentration of skin homogenates is determined with BCA assay following the manufacturer's instruction. Below is a shortened version.

8. Sample and reagent preparation.
   a. Dilute BSA stock with PBS to prepare BSA standard samples at 2,000, 1,500, 1,000, 750, 500, 250, 125, 25, and 0 μg/mL.
   b. Mix 20 μL skin homogenates with 60 μL PBS to prepare a 1:4 fold dilution.

   Note: 1:4 fold dilution reduces 8 M Urea to 2 M, which is within the compatible urea range for BCA assay. This volume (80 μL) is sufficient for a duplicate measurement.

   c. Prepare a sufficient volume of working reagent by mixing 50 parts reagent A with 1 part reagent B following the formula provided by the manufacturer.
9. Microplate procedures.
   a. Add 25 μL standard and skin homogenate samples in duplicate to 96-well plates.
   b. Add 200 μL working reagent to each standard and skin sample wells.
   c. Incubate microplates at 37°C for 30 min (troubleshooting 1).

d. Read absorbance at 562 nm in a microplate reader.
e. Plot standard curve and obtain protein concentrations of skin homogenates by taking into consideration of the dilution factor (troubleshooting 2).

Note: The associated software of a modern microplate reader (for example, SpectraMax iD3 microplate reader with SoftMax Pro) usually allows the input of standard sample concentrations to create standard curves for calculation of protein concentrations of unknown samples in the same software. Users need to select the best-fit standard curve to obtain accurate protein concentrations of unknown samples.

Pause point: Skin homogenates can be stored at −80°C for several months.

Protein digestion

Timing: 5–6 h

Proteins are subjected to reduction, alkylation, precipitation, and resuspension followed by two rounds of digestion with TPCK-treated trypsin according to a prior protocol with slight modifications.[12]

10. Sample preparation.
    a. Identify the skin sample with the lowest protein concentration.
    b. Calculate the volume of other skin homogenate samples to give the same amount of total protein with 400 μL skin sample identified in step 10a.
    c. Calculate the volume of 8 M Urea to make up a total volume of 400 μL for each sample.
    d. Prepare 400 μL skin samples of equal concentrations in new Eppendorf tubes.

Optional: Sample volume can be adjusted to prepare a total 50–200 μg total protein to digest. Bovine Serum Albumin (BSA, 2 μL of 1 mg/mL) can be added as an internal control for MS data normalization.[13]

11. Protein reduction and purification.
    a. Perform protein reduction for skin homogenates by adding 25 μL of 100 mM DTT and incubate at 35°C in a shaking water bath at 100 rpm for 30 min.

⚠ CRITICAL: Wear protective gloves, protective clothing and protective eyeglasses when preparing DTT because it is irritating to eyes and skin.

Note: Prepare the water bath at 35°C in advance. Make sure the power switch is turned on, enough water is added, and the temperature is set at 35°C the night before the procedure.

    b. Alkylate skin homogenates by adding 25 μL of 200 mM iodoacetamide (IAA) and incubate in the dark at 25°C for 30 min.

⚠ CRITICAL: IAA has acute toxicity and respiratory sensitization. Use IAA only in a chemical fume hood and immediately change contaminated clothing. Always use equipment for eye/face protection and wear protective gloves and clothing when using IAA.

Note: IAA is unstable and light-sensitive. Prepare solutions immediately before use and perform alkylation in the dark.

    c. Add cold methanol:chloroform:water mixture (2:1:1, v/v/v) to precipitate proteins.

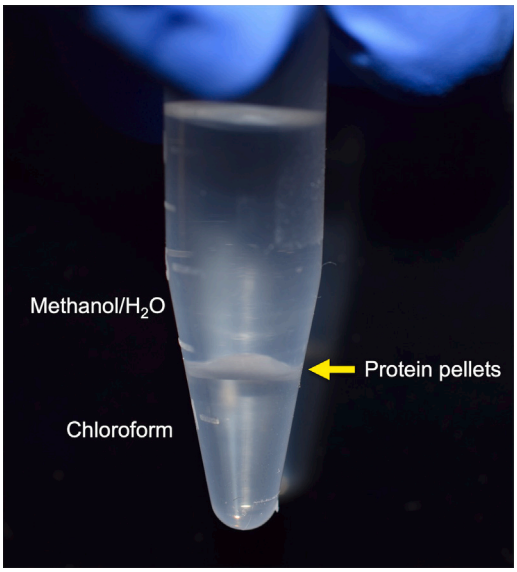


Figure 3. Interphase pellet (arrow) forms between upper methanol/H_2O and lower chloroform phases after Methanol:Chloroform:Water precipitation

⚠ CRITICAL: Methanol is highly flammable and toxic. Use methanol in a chemical fume hood and use equipment for eye/face protection and wear protective gloves and clothing.

⚠ CRITICAL: Chloroform has acute toxicity, skin and eye irritation, carcinogenicity and other organ toxicities. Use chloroform in a chemical fume hood and use equipment for eye/face protection and wear protective gloves and clothing when handling.

Note: Mix methanol: chloroform: water mixture well before adding to your samples since these solvents are immiscible.

d. Shake the tubes vigorously and centrifuge at 16,000 × *g* for 5 min at 10°C.

Note: The white pellets form at the interface of methanol/water (upper) and chloroform (lower), as shown in Figure 3.

e. Carefully remove upper and lower phases with micropipettes without touching pellets (troubleshooting 3).

Note: A small volume of liquid can be kept to avoid the removal of protein pellets.

f. Wash pellets by adding 500 μL ice-cold methanol.
g. Vortex the tube and then centrifuge at 16,000 × *g* for 5 min at 10°C.

⚠ CRITICAL: Methanol is highly flammable and toxic. Use methanol in a chemical fume hood and use equipment for eye/face protection and wear protective gloves and clothing when handling methanol.

Note: Wash the pellets in cold methanol as increased temperature may cause protein loss. This step removes the organic solvent from the previous step. There is no need to break the pellet.

h. Carefully remove methanol after centrifuge.

12. Protein digestion.
    a. Resuspend the pellets in 125 μL 50 mM ammonium bicarbonate (pH 8.0) supplemented with 3% w/v sodium deoxycholate.
    b. Digest protein samples by adding 12.5 μL of 1 mg/mL TPCK-treated trypsin and mix.
    c. Transfer 135 μL samples into digestion tubes and run in a barocycler.

Note: Label the sample# on the digestion tubes clearly for sample identification. Barocycler is an instrument designed for high pressure applications such as accelerated enzymatic digestion of proteins. The barocycler program needs to be set up in advance and the water bath for digestion needs to be set at 35°C at least 20 min before running.

    d. Run the barocycler at 35°C for 75 cycles with 60-s pressure cycle (50-s high pressure, 10-s ambient pressure, 25 kpsi).
    e. Add another 12.5 μL of 1 mg/mL TPCK-treated trypsin into the same tube and the digestion is repeated in the same condition.

13. Sodium deoxycholate removal.
    a. Transfer 145 μL digested samples to new Eppendorf tubes.
    b. Add 15 μL acetonitrile: water (1:1, v/v) containing 5% formic acid into each tube to precipitate sodium deoxycholate.

⚠ CRITICAL: Formic acid is flammable and harmful and causes severe skin burn and eye damage. Use formic acid under a chemical fume hood and wear personal protective gloves, clothing and eye/face protection.

⚠ CRITICAL: Acetonitrile has acute toxicity and eye irritation. Wear personal protective gloves, clothing and eye/face protection and ensure adequate ventilation when handling acetonitrile.

    c. Vortex to mix (troubleshooting 4).

Note: White precipitations form immediately after mixing.

    d. Centrifuge at 11,000 × *g* at 10°C for 5 min.
    e. Carefully collect 80 μL supernatants using micropipettes and add to new Eppendorf tubes.
    f. Centrifuge again at 11,000 × *g* at 10°C for 5 min to ensure no pellets are left in the supernatants.
    g. Transfer supernatants to LC-MS/MS vials and store supernatants at −20°C or inject 25 μL for LC-MS/MS analysis as previously described.[1]

Pause point: The peptides can be stored at −20°C for several weeks.

EXPECTED OUTCOMES

Protein concentration

Skin weight varies within and between groups with the smallest to be 24.2 mg and the highest to be 82.2 mg according to our study. This corresponds to the use of at least 12 μL urea buffer per mg skin tissue since 1000 μL urea buffer is used to homogenize skin tissues including the skin with the maximal weight of 82.2 mg (1000 μL/82.2 mg equivalent to 12 μL per mg tissue weight). Considering skin contains a significant amount of dead tissues that cannot be lysed, the effective volume of lysis buffer per mg skin tissue that can be lysed is expected to be far more than 12 μL. The total protein concentration of the skin is in the range of 1000–3000 μg/mL (Table 1). As expected, a good

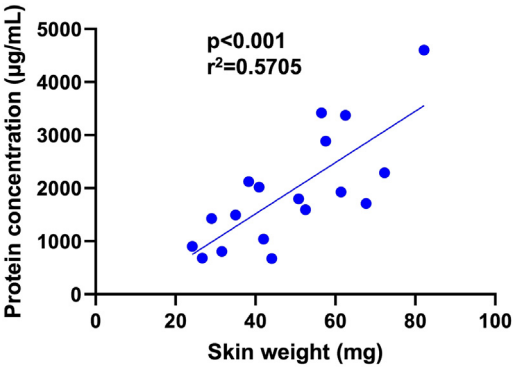


Figure 4. Correlation between protein concentration and skin weight

Data of all groups are pooled for the correlation analysis (n = 18).

correlation is found between protein concentration and skin weight (Figure 4). The good linear correlation hints 1 mL lysis buffer is sufficient to lyse even the largest skin collected in our study. Or else, a gradually reducing protein concentration is expected with increasing tissue weight.

Total proteins detected by LC-MS/MS

In our study, trypsin-digested peptides are separated and eluted using a 180-min gradient from 98:2 to 70:30 buffer A:B ratio (A: 0.1% formic acid in H_2O; B: 0.1% formic acid in acetonitrile) on an Acquity UPLC Peptide BEH C18 column (1.7 µm resin, 2.1 × 150 mm) (Waters Corp., Milford, MA, USA). Eluted peptides are ionized by electrospray (5.5 kV) followed by mass spectrometric analysis on a Sciex 5600 TripleTOF mass spectrometer (Sciex, Framingham, MA, USA). MS data were acquired using Analyst TF 1.7.1 software over a range of 300–1250 m/z followed by data-independent analysis (DIA) as reported.[12,14] Proteins were identified by database search using Spectronaut with less than 1.0% false discovery rate (FDR) and containing 1–3 peaks. It's expected 1500–2000 proteins to be detected in skin samples prepared following the current protocol. The number of proteins detected is expected to differ among different adjuvant treatments. Alum adjuvant tends to reduce the total number of proteins, while MPL and AddaVax tend to increase the total number of proteins to be detected (Figure 5). In our case, physical RFA minimally changes the total number of proteins to be detected when compared to Sham treatment (Figure 5).

Differentially induced proteins

Protein expression levels are compared between adjuvant and Sham groups. Significantly increased or reduced proteins ($p < 0.05$) are further analyzed to identify differentially expressed proteins (DEPs) with at least 30% expression level changes.[15] Different DEPs are expected to be observed in different adjuvant groups. In our previous study, compared with Sham group, AddaVax induced the most protein expression changes (25.2%) and physical RFA induced the least protein expression changes (6.6%) (Table 2). When comparing the percentages of DEPs with at least 30% changes, MPL was found to mainly increase protein expression rather than suppress protein expression (10.6% vs. 1.9%) (Table 2). AddaVax also preferentially increased rather than suppressed protein expression (16.5% vs. 8.7%) (Table 2). In contrast, alum adjuvant was found to preferentially suppress protein expression rather than increase it (3.9% vs. 6.8%) (Table 2). Physical RFA also preferentially suppressed rather than induced protein expression (2.8% vs. 3.8%) (Table 2). The DEPs can be used to analyze uniquely expressed proteins induced by each adjuvant to facilitate the identification of potential molecular mechanisms of vaccine adjuvants.

LIMITATIONS

Mouse skin is very thin with ~200–400 µm in thickness.[16] The thin skin tissue poses a big challenge for accurate intradermal adjuvant delivery. Although Mantoux method is used to guide the intradermal injection,[11] we cannot guarantee 100% adjuvant delivery to the dermal tissue of the skin. Since

Table 2. DEPs with at least 30% change in expression levels

	Increased		Reduced		Total	
	Number	Percentage	Number	Percentage	Number	Percentage
RFA	52	2.8%	70	3.8%	122	6.6%
Alum	60	3.9%	103	6.8%	163	10.7%
MPL/Alum	96	6.0%	118	7.4%	214	13.4%
MPL	204	10.6%	36	1.9%	240	12.5%
AddaVax	323	16.5%	170	8.7%	493	25.2%

Cited from ref. [1] with slight modification of table format.

our labeling is based on the edge of skin blebs after injection, we expect some variations of the marked skin area between individual mice. Furthermore, due to the highly flexible skin that can easily change shape and morphology, tissue collection by cutting along the marked region may also generate variations. Taken together, all these factors are likely responsible for the variations of skin weight observed in our study. A better practice may be marking the center of the injection and then expanding it to include the same skin area (circular or square) that just encloses injection-caused blebs across groups. This practice might help to reduce the variations of skin weight. The accurate labeling and collection of adjuvant-treated skin or other tissues, such as muscle, remain a challenge. Yet, we don't believe skin weight variation observed in our study significantly impacts the identification of DEPs since the same amount of proteins are used in downstream processing although the collection of bigger skin (larger than adjuvant-occupied) is expected to reduce the protein level changes.

TROUBLESHOOTING

Problem 1

In BCA assay, the reaction product shows a color other than an expected purple color (step 9).

Potential solution

Unexpected reaction product color in BCA assay can be caused by a too-high concentration of urea. BCA assay is a colorimetric measurement of total protein concentrations. A high urea concentration over the tolerable range (3 M) interfere with the accurate protein concentration measurement and downstream steps. To solve the problem, make sure the protein sample is properly diluted to contain less than 3 M urea.

Problem 2

Low protein concentrations measured by BCA assay (step 9).

Potential solution

- Make sure the skin was pre-cut into small pieces before homogenization.
- Increase the number of strokes and use more force for each stroke to make sure the skin is pressed against the glass container with little gaps in between.

Problem 3

After cold methanol:chloroform:water precipitation, very little precipitates form (step 11).

Potential solution

- One possible reason can be the high temperature of the solvents, which can cause protein loss. To solve the problem, keep the solvents on ice for sufficient time in advance.

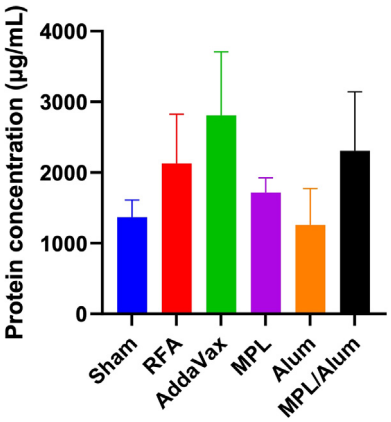


Figure 5. Total number of proteins

The lateral dorsal skin of mice was subjected to RFA or Sham treatment or intradermally injected with AddaVax, MPL, Alum, MPL/Alum. Skin was collected 18 h later and processed for SWATH-MS analysis. The total number of proteins detected was shown for each group (n = 3). Data were expressed as mean ± SEM. Cited from ref [1].

- Another possible reason can be the lack of homogeneity of the solvent mixture. Chloroform is immiscible with water and methanol. Make sure to properly prepare the solvent mixture before use.
- Low protein yield from previous steps could also lead to the same problem, which can be solved by solutions to problem 2.

Problem 4

After adding formic acid, pipette tips are blocked by white precipitates, hindering mixing using pipettes (step 13).

Potential solution

During this step, formic acid is used to precipitate sodium deoxycholate. The white precipitates usually form immediately after the pH is reduced, which is after adding formic acid here. To solve the problem, try mixing by vortexing instead of pipetting up and down.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xinyuan Chen (xchen14@uri.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets.

ACKNOWLEDGMENTS

This work is supported by the National Institutes of Health grants AI139473 (to X.Y.C.). The microplate reader used in this work was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health grant P20GM103430. We thank Dr. Nicholas DaSilva and Juliana Agudelo for their support on the development of this protocol. We would also like to thank Xinliang Kang for her support with the photographs.

AUTHOR CONTRIBUTIONS

Conceptualization, X.Y.C.; Methodology, Y.B.L.; Investigation, Y.B.L.; Writing – Original Draft, Y.B.L.; Writing – Review & Editing, X.Y.C.; Funding Acquisition, X.Y.C.; Supervision, X.Y.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Li, Y., Li, Z., and Chen, X. (2023). Comparative tissue proteomics reveals unique action mechanisms of vaccine adjuvants. iScience *26*, 105800. https://doi.org/10.1016/j.isci.2022.105800.

2. Cao, Y., Zhu, X., Hossen, M.N., Kakar, P., Zhao, Y., and Chen, X. (2018). Augmentation of vaccine-induced humoral and cellular immunity by a physical radiofrequency adjuvant. Nat. Commun. *9*, 3695. https://doi.org/10.1038/s41467-018-06151-y.

3. Li, Y., Li, Z., Zhao, Y., and Chen, X. (2021). Potentiation of recombinant NP and M1-induced cellular immune responses and protection by physical radiofrequency adjuvant. Vaccines *9*, 1382. https://doi.org/10.3390/vaccines9121382.

4. Li, Z., Kim, K.H., Bhatnagar, N., Park, B.R., Jeeva, S., Jung, Y.J., Raha, J., Kang, S.M., and Chen, X. (2022). Physical radiofrequency adjuvant enhances immune responses to influenza H5N1 vaccination. FASEB J *36*, e22182. https://doi.org/10.1096/fj.202101703R.

5. Li, Z., Kang, X., Kim, K.H., Zhao, Y., Li, Y., Kang, S.M., and Chen, X. (2022). Effective adjuvantation of nanograms of influenza vaccine and induction of cross-protective immunity by physical radiofrequency adjuvant. Sci. Rep. *12*, 21249. https://doi.org/10.1038/s41598-022-25605-4.

6. McKee, A.S., and Marrack, P. (2017). Old and new adjuvants. Curr. Opin. Immunol. *47*, 44–51. https://doi.org/10.1016/j.coi.2017.06.005.

7. Reed, S.G., Orr, M.T., and Fox, C.B. (2013). Key roles of adjuvants in modern vaccines. Nat. Med. *19*, 1597–1608. https://doi.org/10.1038/nm.3409.

8. Di Pasquale, A., Preiss, S., Tavares Da Silva, F., and Garçon, N. (2015). Vaccine adjuvants: from 1920 to 2015 and beyond. Vaccines *3*, 320–343. https://doi.org/10.3390/vaccines3020320.

9. Li, Z., Zhao, Y., Li, Y., and Chen, X. (2021). Adjuvantation of influenza vaccines to induce cross-protective immunity. Vaccines *9*, 75. https://doi.org/10.3390/vaccines9020075.

10. Chen, X. (2023). Emerging adjuvants for intradermal vaccination. Int. J. Pharm. *632*, 122559. https://doi.org/10.1016/j.ijpharm.2022.122559.

11. Sticchi, L., Alberti, M., Alicino, C., and Crovari, P. (2010). The intradermal vaccination: past experiences and current perspectives. J. Prev. Med. Hyg. *51*, 7–14.

12. Jamwal, R., Barlock, B.J., Adusumalli, S., Ogasawara, K., Simons, B.L., and Akhlaghi, F. (2017). Multiplex and label-free relative quantification approach for studying protein abundance of drug metabolizing enzymes in human liver microsomes using SWATH-MS. J. Proteome Res. *16*, 4134–4143. https://doi.org/10.1021/acs.jproteome.7b00505.

13. Marques, E., Pfohl, M., Auclair, A., Jamwal, R., Barlock, B.J., Sammoura, F.M., Goedken, M., Akhlaghi, F., and Slitt, A.L. (2020). Perfluorooctanesulfonic acid (PFOS) administration shifts the hepatic proteome and augments dietary outcomes related to hepatic steatosis in mice. Toxicol. Appl. Pharmacol. *408*, 115250. https://doi.org/10.1016/j.taap.2020.115250.

14. Jamwal, R., Topletz, A.R., Ramratnam, B., and Akhlaghi, F. (2017). Ultra-high performance liquid chromatography tandem mass-spectrometry for simple and simultaneous quantification of cannabinoids. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. *1048*, 10–18. https://doi.org/10.1016/j.jchromb.2017.02.007.

15. Mann, M., and Kelleher, N.L. (2008). Precision proteomics: the case for high resolution and high mass accuracy. Proc. Natl. Acad. Sci. USA *105*, 18132–18138. https://doi.org/10.1073/pnas.0800788105.

16. Calabro, K., Curtis, A., Galarneau, J.R., Krucker, T., and Bigio, I.J. (2011). Gender variations in the optical properties of skin in murine animal models. J. Biomed. Opt. *16*, 011008. https://doi.org/10.1117/1.3525565.