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Native Polyacrylamide Gel Electrophoresis Immunoblot Analysis of Endogenous IRF5 Dimerization

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Dear editors,

Please find attached the resubmission of our manuscript entitled "Native PAGE Immunoblot Analysis of Endogenous IRF5 Dimerization". We have taken the suggestions of the editors and reviewers and improved the manuscript. The main changes include an expanded Introduction and Discussion sections, where important background and implications relevant to this protocol are discussed. In addition, we added a new **Figure 2** that addresses the issue of specificity of the antibody, and further demonstrates the robustness of this assay. A separate response to reviewers document addresses the reviewers' concerns and critiques point by point. We believe the manuscript is significantly improved, and we thank the reviewers for all the valuable insights.

Please do not hesitate to contact us if further changes are needed.

Sincerely,

Kwan T Chow
Assistant Professor

TITLE:

Native Polyacrylamide Gel Electrophoresis Immunoblot Analysis of Endogenous IRF5 Dimerization

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KEYWORDS:

native PAGE, gel electrophoresis, IRF5, plasmacytoid dendritic cells, dimerization, interferon regulatory factors

SUMMARY:

A native Western blot method for analyzing endogenous interferon regulatory factor 5 dimerization in the CAL-1 plasmacytoid dendritic cell line is described. This protocol can be applied to other cell lines as well.

ABSTRACT:

Interferon regulatory factor 5 (IRF5) is a key transcription factor for regulating the immune response. It is activated downstream of the Toll-like receptor myeloid differentiation primary response gene 88 (TLR-MyD88) signaling pathway. IRF5 activation involves phosphorylation, dimerization, and subsequent translocation from the cytoplasm into the nucleus, which in turn induces the gene expression of various pro-inflammatory cytokines. A detection assay for IRF5 activation is essential to studying IRF5 functions and its relevant pathways. This article describes a robust assay to detect endogenous IRF5 activation in the CAL-1 human plasmacytoid dendritic cell (pDC) line. The protocol consists of a modified nondenaturing electrophoresis assay that can distinguish IRF5 in its monomer and dimer forms, thus providing an affordable and sensitive approach to analyze IRF5 activation.

INTRODUCTION:

Interferon regulatory factor 5 (IRF5) is an important transcription regulator that plays a prominent role in regulating the immune response, particularly in the release of pro-inflammatory cytokines and type I interferons (IFNs)[1-3]. Misregulation of IRF5 is a contributing factor in numerous autoimmune diseases, as evident by various polymorphisms in the IRF5 locus that are associated with systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis,

etc.[4-10]. Therefore, a robust detection assay for endogenous IRF5 activation state is crucial for understanding the regulatory pathways and downstream effects of IRF5 in a physiologically relevant cellular context.

IRF5 is constitutively expressed in monocytes, dendritic cells (DCs), B cells, and macrophages[1,11]. As with other IRF family transcription factors, IRF5 resides in the cytoplasm in its latent state. Upon activation, IRF5 is phosphorylated and forms homodimers, which then translocate into the nucleus and bind to specific regulatory elements of genes encoding type I IFNs and pro-inflammatory cytokines, eventually inducing the expression of these genes[1,2,11-13]. IRF5 regulates the innate immune responses downstream of various Toll-like receptors (TLRs), such as TLR7, TLR 8, and TLR 9, which are localized in endosomes and use MyD88 for signaling[1,11,14]. These TLRs primarily recognize foreign nucleic acid species such as single-stranded RNA (ssRNA) and unmethylated CpG DNA that are symptomatic of an infection[15-18]. IRF5 has been shown to regulate immune responses against bacterial, viral, and fungal infections[19-21]. Considering IRF5's influential and diverse role in the immune system, enhancing or dampening IRF5 activity could serve as a novel avenue for the development of therapeutic agents[22]. Hence, it is critical to develop a protocol to monitor the activation status of endogenous IRF5 to allow thorough investigation of the pathways and mechanisms regulating IRF5 activity in different cell types.

To the best of our knowledge, no biochemical or gel electrophoretic assay for endogenous IRF5 activation has been published prior to the development of this protocol. Phosphorylation has been shown to be an important first step of IRF5 activation, and a phosphospecific IRF5 antibody was developed that led to the discovery and confirmation of a serine residue important for IRF5 activity[13]. However, while the antibody clearly detects phosphorylated IRF5 when immunoprecipitated or overexpressed[23], it fails to detect IRF5 phosphorylation in a whole cell lysate in our hands (data not shown). Dimerization is the next step of IRF5 activation, and many important studies to date investigating this step relied on overexpression of epitope-tagged IRF5, often in irrelevant cell types that do not normally express IRF5[11,12,24,25]. Previous studies have shown that dimerized IRF5 may not always translocate into the nucleus and hence is not necessarily fully activated[25,26]. An assay for endogenous IRF5 nuclear localization was developed to assess IRF5 activation by imaging flow cytometry[27]. This assay has been applied in studies that were crucial to understanding IRF5 activity, especially in primary or rare cell types[28,29] and greatly advanced the knowledge in the field. However, this assay relies on a specialized instrument that is not widely available to researchers. Further, it is often necessary to investigate the initial steps of activation while dissecting IRF5 regulatory pathways and identifying upstream regulators and pathway components. This study provides a robust and reliable biochemical assay for the early activation events of IRF5 that can be performed in labs equipped with molecular biology tools. The protocol described here will be very useful in investigating the pathways and mechanisms of IRF5 actions, especially when combined with orthogonal assays such as the imaging flow cytometric analysis of IRF5 nuclear localization[23,27,28,30].

Native polyacrylamide gel electrophoresis (native PAGE) is a widely used method to analyze protein complexes[31,32]. Unlike sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE separates proteins on the basis of their shape, size, and charge. It also retains

native protein structure without denaturation[31,33-35]. The protocol presented takes advantage of these features of native PAGE and detects both monomeric and dimeric forms of IRF5. This method is particularly important for detecting early activation events because there is no suitable commercially available antibody that can detect endogenous phosphorylated IRF5. Previously, several published studies used native PAGE to assess IRF5 dimerization. However, the majority of these studies depended on the overexpression of exogenous epitope-tagged IRF5 to analyze activation status[2,13,24,36,37]. This work presents a step-by-step protocol for analyzing endogenous IRF5 dimerization via a modified native PAGE technique in a human plasmacytoid dendritic cell (pDC) line, where IRF5 activity has been shown to be crucial for its function[1,38-40]. This same technique has been applied to other cell lines[23].

PROTOCOL:

NOTE: The protocol described here uses CAL-1 pDC cell line treated with resiquimod (R848), an agonist for TLR7/8. This protocol has been applied to other human and murine cell types, including RAW 264.7 (murine macrophage line), THP-1 (human monocytic cell line), BJAB (human B cell line), Ramos (human B cell line), and MUTZ-3 (human dendritic cell line)[23].

1. Stimulation of CAL-1 cells

1.1. Maintain CAL-1 cell cultures in a T75 flask at 37 °C and 5% CO_2 under sterile conditions with 20−25 mL of RPMI 1640 medium containing 5% fetal bovine serum (FBS), 25 mM HEPES, and 1x mercaptoethanol (i.e., complete RPMI 1640 medium).

1.2. Transfer the cells into a 50 mL conical tube.

NOTE: CAL-1 cells are non-adherent. For adherent cell types, standard trypsinization can be performed to harvest cells.

1.3. Centrifuge the cells at 200 x *g* for 5 min at room temperature (RT). Remove supernatant and resuspend the cell pellet in 8 mL of the complete RPMI 1640 medium to obtain a homogeneous single cell suspension.

1.4. Count the cells using a hemocytometer. Seed the cells at a density of 1 x 10^6 cells per well in a 6 well plate with 4 mL of preheated complete RPMI 1640 medium in each well. Incubate for 20−24 h at 37 °C and 5% CO_2 to allow the confluency to reach 90%−95% (corresponding to approximately 1.5 x 10^6 cells).

1.5. Stimulate the cells by adding 4 µL of 1 mg/mL R848 per well of the 6 well plate (final concentration of 1 µg/mL). Also set up an unstimulated control well with cells without the R848 treatment.

1.6. Ensure the R848 is evenly dispersed by gently rocking the plate side to side. Then, incubate the cells for 2−16 h in the incubator at 37 °C and 5% CO_2.

2. Extraction of cellular proteins

2.1. Transfer the cell suspensions from the 6 well plate into 5 mL centrifuge tubes.

2.2. Centrifuge at 200 x *g* for 5 min at RT. Remove the supernatant and resuspend the cell pellet in 1 mL of phosphate-buffered saline (PBS) to obtain a homogeneous single cell suspension.

2.3. Transfer the cell suspension into a 1.5 mL centrifuge tube.

2.4. Spin down briefly at 12,000 x *g* for 0.5−1 min at 4 °C and carefully remove the supernatant.

2.5. Prepare the lysis buffer containing 6.25 mL of 1 M Tris-HCl pH 7.4 (final concentration of 25 mM), 7.5 mL of 5 M NaCl (final concentration of 150 mM), 0.5 mL of 0.5 M EDTA (final concentration of 1 mM), 2.5 mL of NP-40 (final concentration of 1%) and 7.5 mL of glycerol (final concentration of 5%) in 250 mL of deionized water (ddH_2O). Add 100x protease inhibitor single-use cocktail to a final concentration of 1x to the lysis buffer just before use. Keep the prepared lysis buffer on ice.

NOTE: The lysis buffer without the protease can be stored at 4 °C.

2.6. Resuspend the cell pellet in 30 µL of ice-cold lysis buffer and mix by pipetting up and down.

2.7. Incubate on ice for 15−20 min.

2.8. Clarify the lysate by centrifuging at 12,000 x *g* for 15−20 min at 4 °C. Transfer the supernatant into a new prechilled 1.5 mL centrifuge tube. Keep the extracts on ice at all times.

NOTE: Cell lysates can be stored at -20 °C or -80 °C. Do not boil the samples.

2.9. Measure protein concentration using Bradford reagent.

3. Analysis of IRF5 dimerization by native PAGE

3.1. Prepare the upper (-) and lower (+) chamber electrophoresis buffers. The upper chamber buffer consists of 0.3% sodium deoxycholate (NaDOC) in 1x native PAGE running buffer, and the lower chamber consists of only 1x native PAGE running buffer.

NOTE: Prepare a fresh upper chamber buffer for every new run.

3.2. Rinse a 3%−12% native PAGE gel thoroughly with water without distorting the wells. Set the gel into the mini gel tank and remove the comb. Prerun the gel in a 4 °C cold room or on ice at 150 V for 30 min.

NOTE: Prerunning removes excessive ammonia and persulfate ions that can interfere with the running of the gel.

3.3. During the prerun, prepare the samples for loading by mixing the cellular proteins kept on ice with 4x native sample buffer.

3.4. After the prerun, load 10−15 µg of protein with a final volume of 10−15 µL per sample.

NOTE: Overloading of protein can cause smearing.

3.5. Run gel at 85 V for 30 min, then 150 V for 2 h.

3.6. Soak the gel in SDS running buffer (25 mM Tris pH 8.3, 250 mM Glycine, 0.1% SDS) for 30 min at RT.

NOTE: No agitation is required. Occasionally, the intensity of the band may not be proportional to the amount of protein loaded due to inefficient transfer in the presence of deoxycholate (DOC), which mainly affects the monomeric form of IRF5. Soaking the gel in SDS running buffer prior to the transfer solves this problem. The gel is fragile. Handle with extreme care from the bottom (i.e., higher percentage) end of the gel.

4. Immunoblot analysis of IRF5

4.1. Activate the polyvinylidene difluoride (PDVF) membrane by soaking it in methanol for approximately 5 min.

4.2. Make a cut on one corner of the membrane to indicate its orientation. Assemble the transfer sandwich according to the sequential order detailed in the manufacturer's protocol with extra care to ensure that no air bubbles are trapped within.

4.3. Place the transfer cassette into the tank and transfer at 20 V for 1 h on ice.

NOTE: Perform all incubations and washes in subsequent steps with a rocking shaker.

4.4. Remove the membrane from the cassette with plastic forceps after the transfer is completed. Block the membrane in blocking buffer (TBS) for 45 min at RT.

NOTE: TBS buffer with 5% BSA can also be used as a blocking buffer.

4.5. Incubate the membrane with the primary antibody listed in **Table 1**. Wash the membrane with 1x TBST washing buffer (20 mM Tris, pH 7.0, 150 mM NaCl and 0.1% Tween 20) for 3 min while rocking. Repeat the wash 2x.

[Place **Table 1** here]

4.6. Incubate the membrane with the secondary antibody listed in **Table 1**. Wash the membranes for 3 min in 1x TBST washing buffer. Repeat the wash 2x.

4.7. Scan the blot using an appropriate gel documentation system.

REPRESENTATIVE RESULTS:

The immunoblot (IB) with an anti-IRF5 antibody was performed on CAL-1 cells unstimulated or stimulated with 1 µg/mL R848 for 2 h (**Figure 1**). Cell lysates were prepared, and the native PAGE was performed. In unstimulated CAL-1 cells, IRF5 was detected as a single band on the native PAGE, corresponding to its monomeric form. Upon treatment of CAL-1 cells with R848 for 2 h, the level of IRF5 monomer decreased with a concurrent increase in the accumulation of a slowly migrating band that corresponded to the dimeric form of IRF5.

[Place **Figure 1** here]

The immunoblot with anti-IRF5 antibody was performed on IRF5-overexpressing 293T cells untransfected and transfected with various constructs. Cell lysates were prepared and native PAGE was performed (**Figure 2**)**.** No IRF5 was detected in untransfected 293T cells, demonstrating the specificity of the anti-IRF5 antibody. A single band corresponding to monomeric IRF5 was only detected in the 293T cells overexpressing IRF5. When constructs encoding IRF5-activating proteins, including NRIG (constitutively active RIG-I), MAVS, and IKKβ were cotransfected, a slowly migrating band corresponding to the dimeric form of IRF5 appeared. However, NMDA5 (constitutively active MDA5), a related protein to RIG-I, did not induce IRF5 dimerization when cotransfected.

[Place **Figure 2** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Endogenous IRF5 dimerized in CAL-1 cells when stimulated with TLR7/8 agonist. CAL-1 cells were untreated or treated with R848 for the indicated time. Protein samples were resolved by native PAGE and followed by IB using the anti-IRF5 antibody.

Figure 2: Cotransfection of IRF5-activating factors induced IRF5 dimerization in 293T cells. The 293T cells were untransfected (lane 1) or transfected with IRF5 (lane 2) along with various IRF5 regulators (lanes 3−6). Protein samples were resolved by native PAGE and followed by IB using the anti-IRF5 antibody. NRIG = N-terminal of RIG-I; NMDA5 = N-terminal of MDA5. (Originally published in *The Journal of Immunology*. KT Chow, C Wilkins, M Narita, R Green, M Knoll, YM Loo and M Gale Jr. 2018. Differential and Overlapping Immune Programs Regulated by IRF3 and IRF5 in Plasmacytoid Dendritic Cells. *J. Immunol.* 201 (10) 3036-3050. Copyright © 2018 The American Association of Immunologists, Inc.[23])

Table 1: Specifications of the antibodies used in the immunoblotting procedure.

DISCUSSION:

The protocol described here is a modified native PAGE that distinguishes both monomeric and dimeric forms of endogenous IRF5. There have been few studies reporting the detection of endogenous IRF5 activation using the specialized imaging flow cytometry technique[23,27,28,30]. This protocol uses a common technique and commonplace reagents and tools to assess the endogenous IRF5 activation state during the early events of activation. The protocol entails simple modifications to a standard native PAGE protocol to enable distinction between the monomeric and dimeric forms of IRF5. It can be easily adapted to studies using other cell lines[23]. This modified native PAGE protocol can resolve endogenous IRF5 in its two forms clearly without non-specific protein interferences (**Figure 2**). Endogenous IRF5 from unstimulated cells was detected as a clear single band in this gel system, whereas treatment with R848 for 2 h resulted in the appearance of a band corresponding to IRF5 dimers (**Figure 1**).

A native PAGE dimerization assay for IRF3, a similar transcription factor to IRF5 in the same family, has been developed and widely used in the past two decades[32]. Despite extensive testing and troubleshooting, we were unable to apply the same protocol that employs the Laemmli Tris-glycine system to resolve IRF5 monomer and dimer. The protocol described in this article uses Bis-Tris gradient gels, which have a very different chemistry to the Tris-glycine single percentage gels used in the IRF3 protocol. The different pH and chemical composition of the gel electrophoretic systems may be crucial in distinguishing the various forms of IRF3 and IRF5. Indeed, IRF3 and IRF5, while similar, have different properties (e.g., isoelectric points and modification sites) likely resulting in different behavior while being separated on different gel systems.

A 1x native PAGE running buffer containing DOC was used for the gel run. The buffer needs to be prepared fresh or kept in a clean and protein-free environment to avoid the appearance of white precipitates clouding the solution in the upper chamber as a result of DOC precipitating non-specific proteins. It is highly recommended that the extracted endogenous IRF5 samples be subjected to the native PAGE as soon as possible, preferably within a week with minimal freeze-thaw cycles. Otherwise, there may be significant protein degradation. The degradation is observed with storage at both -80 °C and -20 °C. In addition, the pH of the SDS running buffer and the TBST washing buffer should be adjusted at RT.

The ideal final volume of sample loaded in each well was 10−15 µL, but slight adjustments might be required depending on different cell types. After the initial run at 85 V for 30 min, it is recommended to continue running the gel at 150 V for approximately 2 to 3 h to attain distinct separation and resolution of the IRF5 monomer and dimer. After the run is finished, it is of utmost importance to handle the gel meticulously from its bottom end due to its differential levels of density, ranging from 3% at the top and increasing towards 12% at the bottom. In this case, it is preferable to remove the gel from the plate by immersing it in the used running buffer, which acts as an impact cushion to minimize friction and allows the gel to float away from the plate to avoid breakage.

A few minor drawbacks of this protocol include the limited selection of gels available to achieve

the desired results. Homemade gels and a few other brands of commercially available gels have been tested without success. In our hands, use of a commercial running buffer and gel system contributed to the robustness and reproducibility of this protocol, although extensive testing of homemade buffers has not been performed. Attention to detail is essential, and experience is key to success in obtaining clear results. Lastly, the resolution of IRF5 required a long time (i.e., 2−3 h) to get an ideal separation of the monomer and dimer. Further enhancement and modifications in the future can improve the efficiency and minimize the drawbacks of this protocol.

In conclusion, this protocol is a robust assay for the detection of endogenous IRF5 monomer and dimer. It is suitable for applications in various human and murine cell types expressing endogenous IRF5. It will be a valuable tool to study the IRF5 regulatory pathways and signaling components in various cell types.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

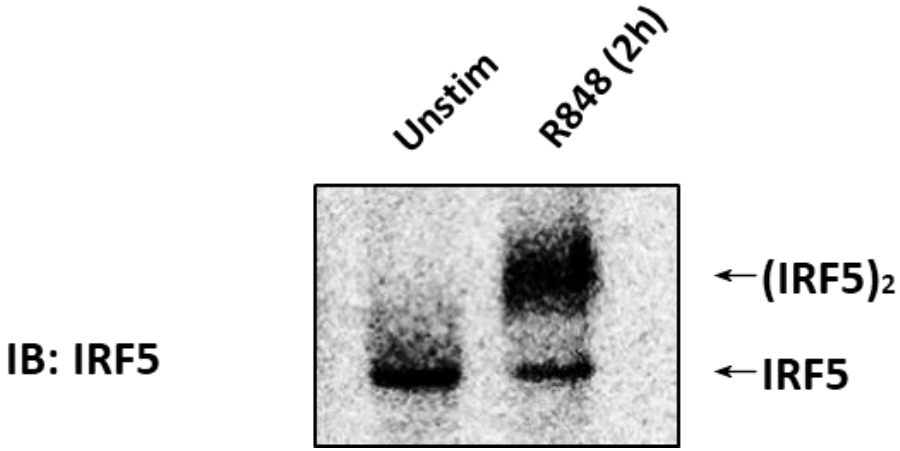
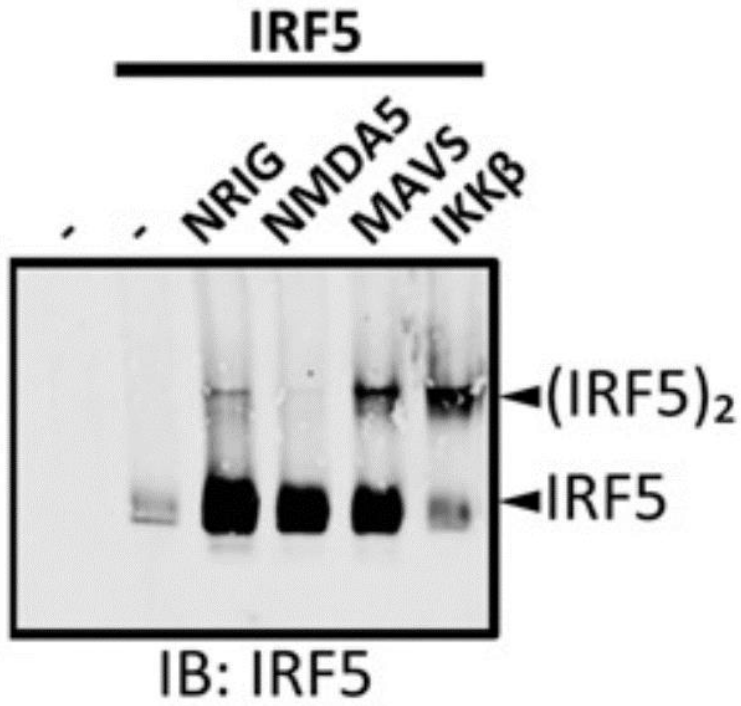


Figure 2

Click here to access/download;Figure;Figure 2.ai



Table

	Dillution	Dillution buffer	Incubation	Comments
Primary antibody (Anti-IRF5)	1/1000	TBS blocking buffer	Overnight at 4 °C or 2 h at RT	Diluted antibodies can be reused several times if stored at 4 °C in the presence of 0.02% sodium azide.
Secondary antibody (Anti-rabbit)	1/10,000	TBS blocking buffer	45 min at RT	Diluted antibodies can be reused several times if stored at 4 °C in the presence of 0.02% sodium azide.
NOTE: Dilution need to be optimized as it varies between manufacturers.				

Table of Materials

Reagent and Equipment	Company	Catalog Number
2-Mercaptoethanol	Life Technologies, HK	21985023
300 W/250 V power supply 230 V AC	Life Technologies, HK	PS0301
Anti-IRF5 antibody	Bethyl Laboratories, USA	A303-385
BIOSAN Rocker Shaker (cold room safe)	EcoLife, HK	MR-12
EDTA Buffer, pH 8, 0.5 M 4 X 100 mL	Life Technologies	15575020
Glycerol 500 mL	Life Technologies	15514011
Glycine	Life Technologies, HK	15527013
Goat anti-Mouse IgG DyLight 800 Conjugated Antibody	LAB-A-PORTER/Rockland, HK	610-145-002-0.5
Goat anti-Rabbit IgG DyLight 800 Conjugated Antibody	LAB-A-PORTER/Rockland, HK	611-145-002-0.5
Halt protease inhibitor cocktail (100x)	Thermo Fisher Scientific, HK	78430
HEPES	Life Technologies, HK	15630080
LI-COR Odyssey Blocking Buffer (TBS)	Gene Company, HK	927-50000
Mini Tank blot module combo; Transfer module, accessories	Life Technologies, HK	NW2000
NativePAGE 3-12% gels, 10 well kit	Life Technologies, HK	BN1001BOX
NativePAGE Running Buffer 20x	Life Technologies, HK	BN2001
NativePAGE Sample Buffer 4x	Life Technologies, HK	BN2003
NP-40 Alternative, Nonylphenyl Polyethylene Glycol	Tin Hang/Calbiochem, HK	#492016-100ML
PBS 7.4	Life Technologies, HK	10010023
Polyvinylidene difluoride (PVDF) membrane	Bio-gene/Merck Millipore, HK	IPFL00010
Protein assay kit II (BSA)	Bio-Rad, HK	5000002
R848	Invivogen, HK	tlrl-r848
RPMI 1640	Life Technologies, HK	61870127
Sodium Chloride	ThermoFisher	BP358-1
Sodium deoxycholate ⩾97% (titration)	Tin Hang/Sigma, HK	D6750-100G
Tris	Life Technologies, HK	15504020
TWEEN 20	Tin Hang/Sigma, HK	#P9416-100ML

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Author(s):	**Meijun WANG, King Hoo LIM, Kwan T CHOW**

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CORRESPONDING AUTHOR

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Title:	**Assistant Professor**
Signature:	
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We have thoroughly proofread the manuscript.

2. Authors and affiliations: Please provide an email address for each author.

We have added the email address for each author in this part.

3. Keywords: Please provide at least 6 keywords or phrases.

We have included the keywords for our manuscript.

4. 2.4: Please specify centrifugation parameters.

We have added these parameters in 2.4.

5. 2.5: Please list an approximate volume of the buffer to prepare.

We have listed the volume in addition to the final concentration of each ingredient for the preparation of buffer in 2.5.

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We have included the relevant permission information with this version of the manuscript.

7. Figure 2: Please change the time unit “hr” to “h”.

We have made the appropriate changes throughout the manuscript.

8. Please combine Table of specific reagents and specific material and equipment into a single worksheet.

We have made the suggested change.

9. Please make the worksheet “Table of recipes” a table and reference the table in the manuscript.

We have made the suggested changes.

10. References: Please do not abbreviate journal titles; use full journal name.

We have edited these journal titles in References.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Native PAGE method to analyze the formation of an endogenous IRF5 dimer in an immortalized cell line

Major Concerns:

1. In the first paragraph of the Introduction, the authors state that their method is the only one to "assay for endogenous IRF5 activation" and then in a later sentence refer to the use of Imagestream imaging flow assays to detect IRF5 nuclear localization. Given that IRF5 is only considered "active" once it translocates from the cytoplasm to the nucleus, the Imagestream assay was the first endogenous assay to measure IRF5 activity. Other Labs, including the Reich Lab and the Hiscott Lab, have shown cases where IRF5 homodimerizes and still does not translocate to the nucleus. As such, this might need to be included in the Intro, as the authors mention that assays might need to be combined to support activation.

We thank the reviewer for highlighting these studies and bringing up an important point. We have included a more in-depth discussion on this point in the Introduction.

2. In the Discussion, line 210, the authors refer to "complications in recent studies" but don't refer to any particular studies. It would be helpful to know what the authors are referring to.

We have made the appropriate changes in the Discussion. Many previous studies assessed IRF5 activation by overexpressing exogenous epitope-tagged IRF5 in sometimes irrelevant cell lines. A phospho-specific antibody was published but failed to detect endogenous IRF5 activation without immunoprecipitation or overexpression.

Our protocol is to our knowledge the only biochemical assay that determines the early event of endogenous IRF5 activation in whole cell lysates.

3. In the Discussion, line 213, the authors suggest that their assay has "high specificity towards IRF5" but the assay itself has little to do with specificity; specificity of the assay is completely dependent on having an antibody that detects IRF5 with high specificity. In this regard, the authors need to show the entire gel with molecular weight markers to help prove specificity. The bands as shown could be anywhere on the gel and may not correlate with appropriate sizes of the IRF5 monomer and dimer. Last, the authors use an antibody that is not a common one in the field of IRF5 research. If it is commonly accepted, it would be helpful to include other refs using this antibody to detect human IRF5, or show specificity of the antibody with positive and negative controls cells.

Thank you reviewer for raising this excellent point. In a previous research paper published by our group (Chow et al. The Journal of Immunology. 2018; 201:3036-3050) where we first introduced the use of this protocol, we demonstrated IRF5 antibody specificity in several figures, including an immunoblot with no band in non-IRF5-expressing cells (293T) and a specific band corresponding to the size of IRF5 in these cells when FLAG-IRF5 was overexpressed. The identity of this single band was confirmed by an anti-FLAG antibody (data not shown). In another study published by our group, we demonstrated that this IRF5 antibody detects an IRF5 specific band in wild type bone marrow derived dendritic cells, but not in knockout cells (Chow et al. Journal of Leukocyte Biology. 2019). Further, we demonstrated that this antibody is ChIP-capable and specific as a single band corresponding to the size of IRF5 is immunoprecipitated by this antibody. We have added a figure from the JI study as the new Figure 2 in this manuscript to illustrate this point.

4. It would be helpful to include a couple of Native gel examples from different cell lines, or better yet, primary cells, to really show that the protocol is working in multiple cell types (immortalized and primary). This would be of extreme value to the field.

We thank the reviewer for this excellent suggestion. Several figures demonstrating that this assay works in with multiple cell lines have previously been published by our group (Chow et al. The Journal of Immunology. 2018; 201:3036-3050). In those figures, IRF5 overexpression in 293T cells was first used to demonstrate the feasibility of this assay, and multiple human and murine cell lines were then tested for IRF5 dimerization upon TLR7/8 stimulation. We have expanded the discussion to highlight these figures.

Minor Concerns:

1. Many references are incorrect; for instance, ref 9 is used to refer to IRF5 immune regulation but is actually an antibody paper. Ref 1, for the Imagestream imaging flow assay, is not the original ref of this method to measure IRF5 activity.

We thank the reviewer for pointing out these mistakes and apologize for the oversight. We have made the appropriate changes.

Reviewer #2:

Manuscript Summary:

This manuscript describes a method of detecting IRF5 activation and dimerisation by native PAGE. Although dimerisation assay by native PAGE is extensively used in viral immunology to monitor the activation status of important transcription factors, this manuscript clearly outlines the steps involved in this method, which should be of interests to the readers, young and also established scientists in fields that uses molecular biology in their research.

Major Concerns:

In the introduction, it should be stated that this method is particularly important when there is not suitable antibody that is specific enough to detect phosphorylated protein of interest, then dimerization assay would be very useful to detect activation. There are a lot of researchers who heavily rely on the antibodies to phosho-proteins and observe non-specific bands and are unsure of their activation status.

We thank the reviewer for raising this important point. We have expanded the Introduction to include a discussion on this point.

Line 74. The author should state what R848 is for the benefit of the readers.

We have made the appropriate change.

Line 116-117, would this be protease and phosphatase inhibitor cocktails or specific protease inhibitors like PMSF...

We use Halt Protease Inhibitor Single-Use Cocktail (100X) from Thermo Fisher Scientific, which contains several inhibitors that inhibit the major classes of proteases but does not include any phosphatase inhibitor. We have included the manufacturer of the protease inhibitors we use in the methods.

Line 137, please describe why pre-run is important to the readers.

Pre-runs are conventionally performed to remove excess ions that may interfere with separation of the proteins. We have added this detail in the protocol.

Line 169, for blocking, skim milk or BSA is preferred?

We use Odyssey Blocking Buffer (TBS) from LI-COR because the background tend to be lower. However we have successfully performed this assay using homemade 5% BSA blocking buffer. We have added this detail in the protocol.

It appears that this protocol is specific to Invitrogens gels, and this is a big drawback of this manuscript, and limits the practicality to other scientists who make their own or use other brands such as bio-rad.

We agree it is an unfortunate drawback, but it is currently the only protocol we have tried that allows us to assay IRF5 monomer and dimer. We have tried but failed to use the Laemlli system (Tris-glycine) that successfully separates IRF3, a related protein. We suspect the Bis-tris gradient system of the Invitrogens native gels offer the appropriate chemistry and pH that allow the specific requirement for separating IRF5. We have added a brief discussion on this point in the discussion.

Minor Concerns:

What is the difference between the methods described in this manuscript and the protocol by Prof. Fujita at Kyoto U (Iwamura T et al. Genes Cells. 2001 Apr;6(4):375-88).

The Fujita protocol is a widely used assay to study IRF3 biology. We have performed the same protocol successfully with Ready Cast gels (as described in the original paper) as well as homemade Tris-glycine 7.5% gels. The key differences between our protocol for IRF5 and the Fujita protocol for IRF3 is the chemistry of the gel system. The Fujita protocol utilizes Tris-glycine gels without SDS, which runs at a different pH than the bis-tris system that our protocol uses. Further, the Fujita protocol utilizes a single percentage gel (7.5%) while our protocol uses a gradient gel (3-12%). These differences highlight the different biology of IRF3 and IRF5, despite them being in the same family of transcription factors. Their different phosphorylation and modification states likely give rise to different isoelectric points and conformation, where one gel system cannot substitute for another in separating their monomer and dimer forms. In fact, we have repeatedly tried using the Fujita protocol for IRF5 and failed to detect the monomer/dimer forms. We have added a discussion on this point in the discussion.

Reviewer #3:

Manuscript Summary:

IRF5 is an important transcription factor in immune cells. IRF5 activation essentially requires dimerization, and therefore determination of this dimerization is important to study IRF5 activity. This manuscript provides a protocol to determine IRF5 dimerization.

The assay seems robust and the protocol in general is clear and easy to follow. My suggestion would be to add a few more details that are currently lacking.

Major Concerns:
No major concerns.

Minor Concerns:
A few minor issues that should be addressed (line numbers indicated):

101. Instead of shaking the plate, would it not be better to resuspend using a pipette?

This is a sensitive cell type that may be aberrantly activated if handled inappropriately, hence we prefer to avoid rigorous pipetting and gently shake the plate instead.

106 (2.1). Are these cells completely non-adherent? If a subpopulation of the cells will stick to the bottom of the well and therefore is not transferred to the tube this could bias the results. If the cells are (partially) sticky, it would be nice if the authors could describe a way to detach the cells.

This specific cell line is non-adherent. We have previously tried other cell lines and standard trypsinization works well. We have added a note in the protocol.

113 (2.4). Please specify the duration, g, and temperature of spinning down the cells.

We have made the appropriate changes throughout the protocol.

115 (2.5). I could not find the concentrations of the protease and phosphatase inhibitors. Please add these.

We use a commercial protease inhibitor (100x) diluted to 1x. We have added this detail in 2.5.

120 (2.6). Please clarify the temperature of the lysis buffer when it is added to the cells.

We have added the details in 2.6.

232. Correct typo: change "outmost" to "utmost".

We thank the reviewer for pointing out the typo. We have made the change.

Figure 2: For clarity, please change the headings above the lanes: not "-" and "2", but "Unstim" and "R848 (2hr)".

We have made the suggested change.

Reviewer #4:

Manuscript Summary:
It is brief but informative.

Major Concerns:
Although it is not a novel technique and the journal does not require novelty, the authors failed to review the large amount of literature where the same procedure has been applied for endogenous IRF3 dimer-monomer resolution. Being all the same, it is likely that a good IRF antibody is the key or the most important component of the technique. Further, although the separation of IRF5 dimer-monomer is evident, it is not that impressive. It would have been more informative additional IRF5 activation ligands, exogenous IRF5 mutants (constitutive active or inactive), etc, to show the versatility, reliability, reproducibility of the assay. In fact, comparison to a well-known IRF3 dimer-monomer assay, that uses the same system, would have been very supportive. An accompanying loading control using Tubulin or Actin antibody can provide a better view on the variation of IRF5 activation.

We thank you reviewer for raising this excellent point. A similar point regarding the similarity between IRF3 and IRF5 and the potential application of the IRF3 dimerization protocol to assaying IRF5 activation was raised by another reviewer.

We are quite familiar with the Fujita protocol (Iwamura T et al. Genes Cells. 2001 Apr;6(4):375-88) that is a widely used assay to study IRF3 activation. We routinely perform IRF3 dimerization assays with this protocol using homemade Tris-glycine 7.5% gels (equivalent to the Ready Cast gels stated in the original paper). While choosing an appropriate IRF5 antibody was extremely important to the success of running this assay, simply applying the IRF3 protocol did not work for IRF5 in our hands despite repeated attempts.

The key differences between our protocol for IRF5 and the Fujita protocol for IRF3 is the chemistry of the gel system. The Fujita protocol utilizes Tris-glycine gels without SDS, which runs at a different pH than the Bis-Tris system that our protocol uses. Further, the Fujita protocol utilizes a single percentage gel (7.5%) while our protocol uses a gradient gel (3-12%). These differences highlight the different biology of IRF3 and IRF5, despite them being in the same family of transcription factors. Their different phosphorylation and modification states likely give rise to different isoelectric points

and conformation, where one gel system cannot substitute for another in separating their monomer and dimer forms. In fact, we have repeatedly tried using the Fujita protocol for IRF5 and failed to detect the monomer/dimer forms.

We agree that additional IRF5 activation ligands and exogenous IRF5 mutants (constitutive active or inactive), etc, would be very informative. Some of these experiments have been performed and previously published by our group (Chow et al., Journal of Immunology 2018; 201:3036-3050) where different TLR ligands and cell lines were tested. We feel that generating and testing exogenous IRF5 mutants are beyond the scope of this manuscript, whose goal is to provide the scientific community a robust assay to study IRF5 activation state. We believe this protocol will allow more in-depth dissection of IRF5 biology in the future.

Because loading controls such as Tubulin or Actin are not conventionally used for native gel electrophoresis, we opted to not include them. We believe that prior counting and seeding of same number of cells, the short treatment duration (2-6 hours), the quantification of protein lysates, and loading of same amount of protein should sufficiently ensure equal loading. We have separately performed SDS-PAGE gel electrophoresis using GAPDH as a loading control to demonstrate that the IRF5 protein level does not change throughout the course of our experiment (data not shown).

We have included these points in the discussion.

Minor Concerns:
The article requires grammar editing and in some parts rewriting of the sentences. The author claims that the detection of IRF5 activation has been a problem in the field and that proposed technique will solve this issue, however there are not references supporting this statement.

We thank you for pointing out this important point. We agree and have made the appropriate changes.

Xiaoyan Cao <xiaoyan.cao@jove.com>

Re: Response Requested: JoVE Submission JoVE60393R1 - [EMID:734ad5268cf1222e]

1 message

Dr. CHOW Kwan <kwan.chow@cityu.edu.hk> Mon, Jul 22, 2019 at 4:29 PM
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On Jul 23, 2019 4:23 AM, Xiaoyan Cao <em@editorialmanager.com> wrote:
CC: "Meijun Wang" meijwang@cityu.edu.hk, "King Hoo Lim" kinglim4@cityu.edu.hk

[EXSCINDED]

Dear Dr. Chow,

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Please feel free to email me with any questions or concerns about your manuscript.

Best Regards,

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