Application Note

INTEGRA

Western blot protocol automation with the ASSIST PLUS pipetting robot and Simple Western™

Introduction

Automated western blotting is a cutting-edge technology revolutionizing traditional protein detection and analysis. Employing robotic systems, microscale separation, and advanced imaging technologies streamlines the labor-intensive and time-consuming process of western blotting. Automating the multiple workflow steps increases efficiency, making it an invaluable tool in various fields of biological research.

Simple Western – developed by ProteinSimple, a Bio-Techne brand – is the only fully automated western blotting solution on the market. The advanced, capillary-based technology enables efficient and accurate high throughput protein separation, detection and quantification, with all assay reagents and samples in 1 plate. Simple Western assays are advancing research and development in many applications, including cancer and immuno-oncology, cell and gene therapy, regenerative medicine and targeted protein degradation.

The ASSIST PLUS pipetting robot and D-ONE single channel pipetting module effortlessly fill plates specifically designed for Simple Western instruments – like Jess™ or Abby™ – allowing 100 % hands-free time without worrying about tedious liquid handling. INTEGRA protocols automate the Simple Western plate set-up for chemiluminescence detection of single target, multi-target (using RePlex™) or total protein assays. This application note describes fully automated liquid handling of all 3 assay types by performing chemiluminescence detection and total protein analysis in 1 RePlex normalization assay. The results show equivalent performance to manual plate filling, with comparable on-deck sample and reagent stability.

Key benefits:

- Full walk-away western blot protocol automation combines the ASSIST PLUS pipetting robot with the D-ONE single channel pipetting module and the Simple Western Jess. On top of that, the operator benefits from VIALAB's flexibility to create sample preparation protocols for the ASSIST PLUS.
- Foolproof liquid handling and plate filling with D-ONE's liquid level detection (LLD) and automated GRIPTIPS® pipette tip selection, ensuring precision and accuracy for both low and high volume transfers.
- VIALAB's labware tool simplifies the labware definition for unique plates, and the D-ONE module can even accommodate irregular well distributions.
- Simple Western advanced capillary electrophoresis immunoassay technology enables reliable, high throughput, automated western blot analysis of up to 24 samples per run, with results ready in as little as 3 hours.
- Simple Western chemiluminescence and NIR/IR fluorescence detection provide flexible multiplex analysis capabilities, and ensure high sensitivity when working with precious samples or low abundance targets. RePlex enables 2 immunoassays in a single capillary, and even provides total protein detection to accurately normalize protein expression data.
- The small footprints of the ASSIST PLUS and Jess instruments save space, so they fit easily into any laboratory.

ASSIST PLUS

Overview: How to fill the Simple Western Jess plate with the ASSIST PLUS

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The ASSIST PLUS pipetting robot and D-ONE single channel pipetting module, together with the Simple Western Jess, automate all the liquid handling steps required to analyze 24 samples, providing a complete walk-away solution for western blot protocol automation.

The whole workflow consists of 2 steps (**Figure 1**):

1. Simple Western Jess plate filling with the D-ONE and the ASSIST PLUS
2. Protein analysis with the Simple Western Jess

This application note provides Jess plate filling protocols for chemiluminescence detection of single targets, multiple targets (using RePlex) and total protein analysis of prepared samples and reagents for reliable downstream protein analysis using Simple Western Technology.

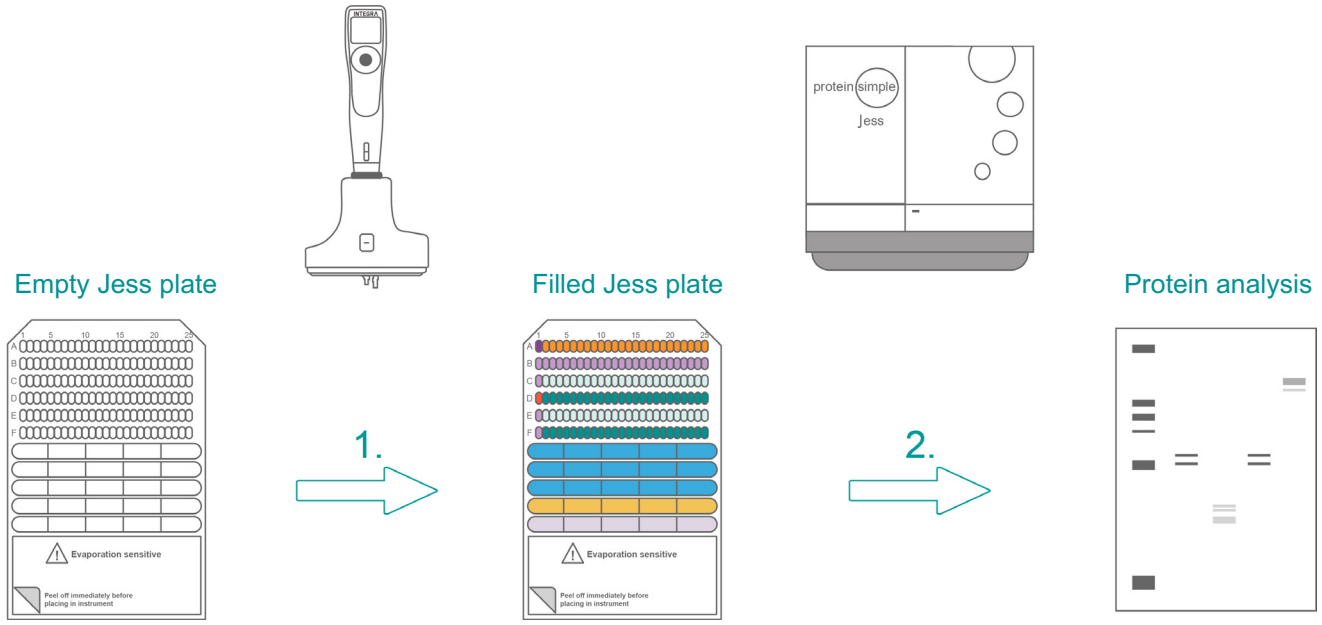

Figure 1: 2-step western blot automation protocol.

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Plate set-up for RePlex chemiluminescence western blot protocol automation

Experimental set-up

Deck Position A: Wash buffer (blue)
Deck Position B: B1 – antibody diluent (lavender), streptavidin-HRP (red), luminol-peroxide mix (yellow), RePlex mix (pink); B2 – primary antibody probe 1 (light green-1), secondary antibody probe 1 (green-1), primary antibody probe 2 (light green-2), secondary antibody probe 2 (green-2); B3 – biotinylated ladder (violet); B4-B6 – prepared samples (orange, arrow indicates processing direction)

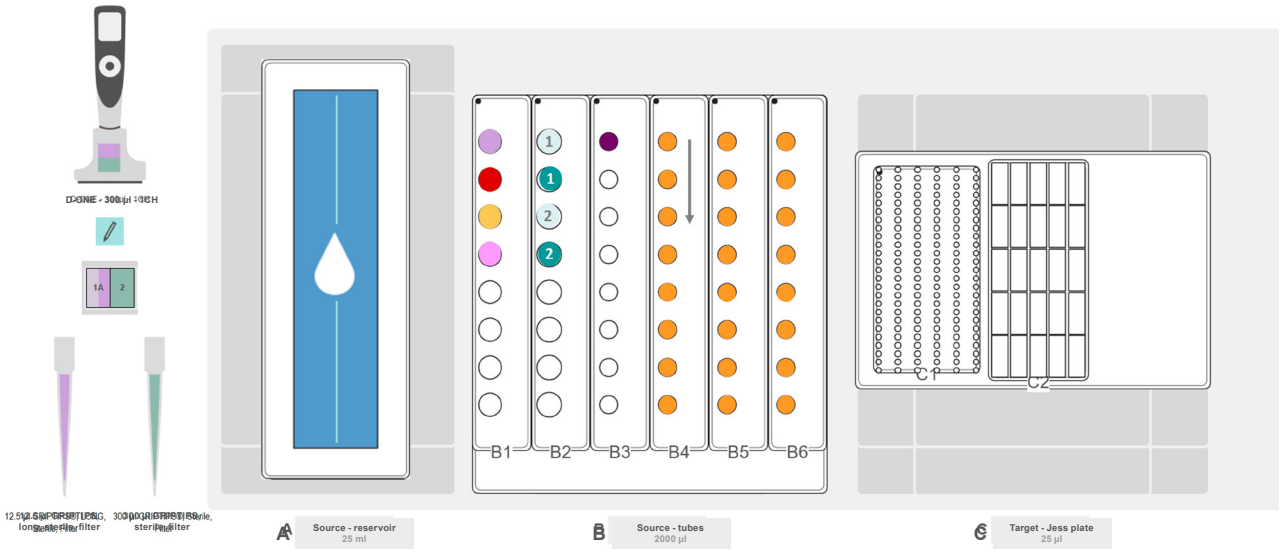

Figure 2: Deck set-up for Jess plate filling to perform RePlex chemiluminescence analysis. **Position A:** Source – 25 ml reservoir. **Position B:** Source – INTEGRA tube rack for 1.5/2 ml (B1-B2) and 0.5 ml (B3-B6) microcentrifuge tubes. **Position C:** Target – Simple Western Jess plate.

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1. RePlex plate set-up

STEP: Jess plate set-up for RePlex.

HOW TO: Place a 25 ml reservoir on deck Position A and fill it with at least 8 ml of wash buffer. Place the INTEGRA tube rack on Position B with the specific reagent and sample tubes, as indicated in the experimental set-up. As shown in **Figure 2**, the Jess plate is in landscape orientation on Position C. LLD enables the use of different aliquot sizes, and the D-ONE informs the operator if the liquid volume is insufficient.

Equip the ASSIST PLUS with the 0.5-300 µl D-ONE, and run the VIALAB program 'Jess_plate_setup_RePlex'. The ASSIST PLUS and D-ONE with 12.5 µl long, sterile, filter GRIPTIPS transfer 10 µl of antibody diluent from a 2 ml tube in Position B1 (**Figure 2**, lavender) to wells B1-B25, C1, E1 and F1 of the Jess plate (**Figure 3**) in Position C. A 1 µl pre- and post-dispense guarantees precise pipetting while preventing bubble creation during dispensing. By automatically changing GRIPTIPS between different reagents or samples, the D-ONE transfers 10 µl of primary antibody probe 1 from a 1.5 ml microcentrifuge tube in B2 (**Figure 2**, light green-1) to wells C2-C25 (**Figure 3**) of the Jess plate in Position C (**Figure 4a**). The D-ONE transfers 10 µl of streptavidin-HRP/NIR from the second 2 ml screw cap vial in B1 (**Figure 2**, red) to well D1 (**Figure 3**). Wells D2 to D25 (**Figure 3**) are filled with 10 µl secondary antibody probe 1 from a 1.5 ml microcentrifuge tube in B2 (**Figure 2**, green-1). 10 µl primary antibody probe 2 is transferred from Position B2 (**Figure 2**, light green-2) to wells E2 to E25 (**Figure 3**), and 10 µl secondary antibody probe 2 is transferred from B2 (**Figure 2**, green-2) to wells F2 to F25 (**Figure 3**).

Using 300 µl sterile, filter GRIPTIPS, the D-ONE transfers 500 µl of wash buffer (**Figure 2**, blue) from the 25 ml reservoir on Position A to the Jess plate compartments in 2 steps (**Figure 4b**), as indicated in **Figure 3**. A slow speed (5) prevents bubble creation during buffer dispensing. Afterwards, 170 µl of luminol-peroxide mix is transferred from the 2 ml tube in B1 (**Figure 5**, yellow), and 300 µl of RePlex reagent mix from another 2 ml tube in B1 (**Figure 5**, pink) to the compartments indicated in **Figure 3**. The D-ONE then transfers the RePlex reagent mix in 2 steps, with a pre- and post-dispense of 10 µl to prevent bubble creation during dispensing.

5 µl of biotinylated ladder is transferred from a 0.5 ml microcentrifuge tube in Position B3 (**Figure 2**, violet) to well A1 (**Figure 3**). As indicated by the arrow in **Figure 2**, the D-ONE transfers 3 µl of prepared sample (**Figure 2**, orange) from each 0.5 ml microcentrifuge tube in Positions B4 to B6 (**Figure 2**, orange) to wells A2-A25 (**Figure 3**). Fast dispensing (speed 10) increases the accuracy for small volumes. The pipette then instructs the user to centrifuge the plate for 5 minutes at 2500 rpm, before proceeding with Simple Western Jess protein separation and immunodetection.

Tips:

- A prompt can be included in the protocol before starting the sample transfer to instruct the user to prepare the samples during reagent transfer.
- VIALAB's simplified programming allows plate set-up to be easily adjusted to perform western blot normalization by replacing the second chemiluminescence detection with a total protein analysis, as indicated in the kit insert.

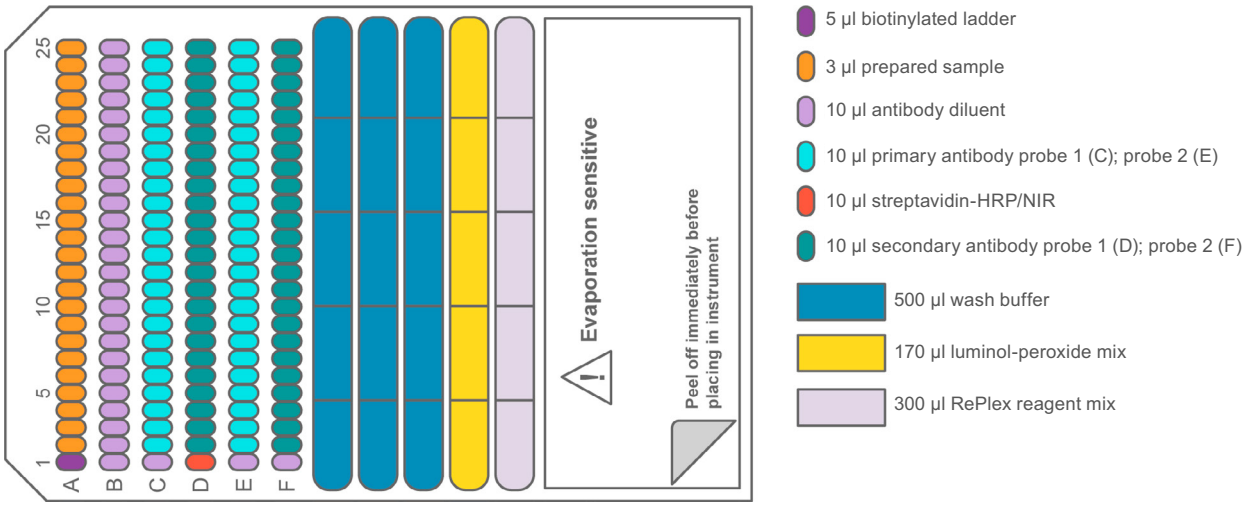
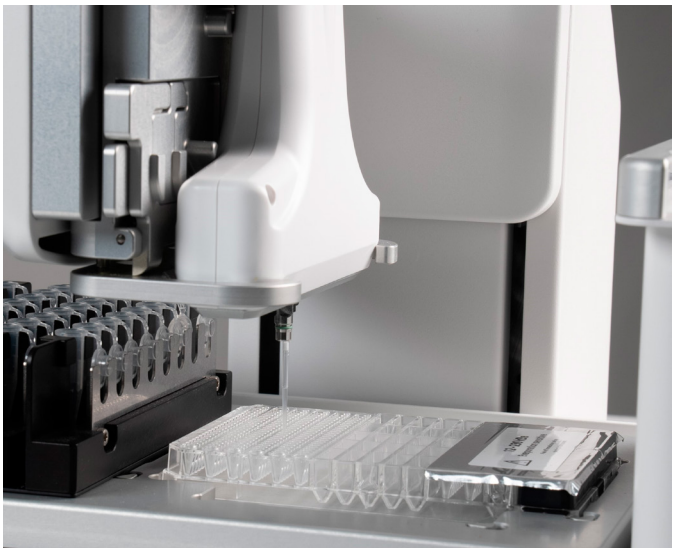


Figure 3: How to fill the Simple Western Jess plate for RePlex chemiluminescence analysis.

a)



b)

Figure 4: The D-ONE transfers a) the primary antibody and b) the wash buffer to the Simple Western Jess plate.

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Plate set-up for chemiluminescence western blot protocol automation

Experimental set-up

Deck Position A: Wash buffer (blue)
Deck Position B: B1 – antibody diluent (lavender), streptavidin-HRP (red), luminol-peroxide mix (yellow); B2 – primary antibody (light green), secondary conjugate (green); B3 – biotinylated ladder (violet); B4-B6 – prepared samples (orange, arrow indicates processing direction)

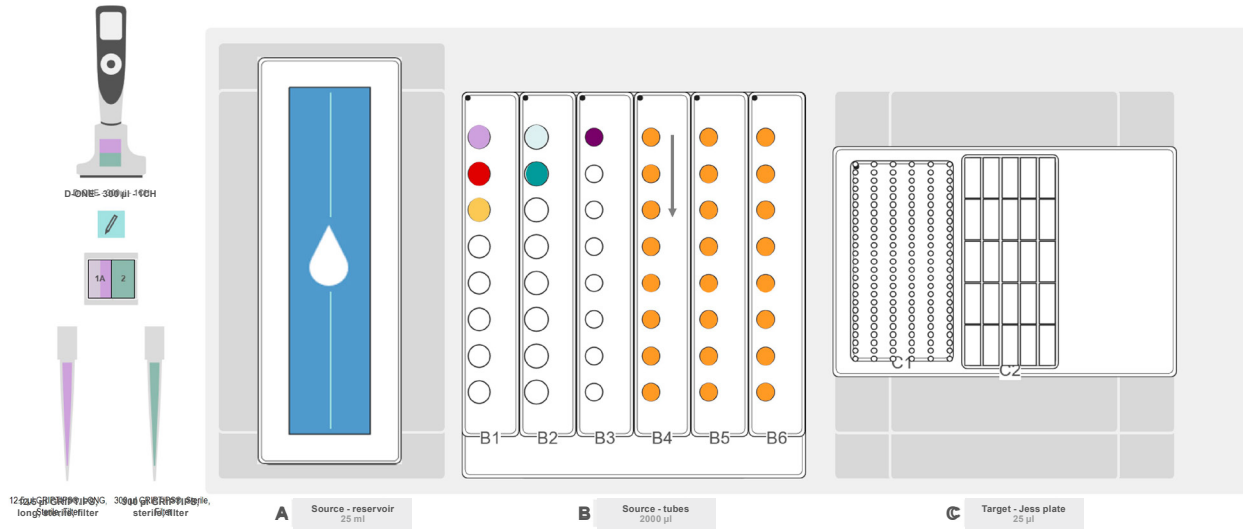

Figure 5: Deck set-up for Jess plate filling to perform chemiluminescence analysis. **Position A:** Source – 25 ml reservoir. **Position B:** Source – INTEGRA tube rack for 1.5/2 ml (B1-B2) and 0.5 ml (B3-B6) microcentrifuge tubes. **Position C:** Target – Simple Western Jess plate.

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2. Chemiluminescence plate set-up

STEP: Jess plate set-up for chemiluminescence western blot.

HOW TO: Prepare a similar deck set-up to the RePlex western blot protocol, but without the RePlex reagent mix or the primary and secondary antibodies for probe 2 (**Figure 5**).

Select and run the VIALAB program 'Jess_plate_setup_chemiluminescence'. Following a similar procedure as for RePlex chemiluminescence detection, the D-ONE starts by transferring 10 µl of antibody diluent (**Figure 5**, lavender) to wells B1-B25 and C1 of the Jess plate (**Figure 6**) in Position C. 10 µl of primary antibody (**Figure 5**, light green) is then transferred to wells C2-C25, 10 µl of streptavidin-HRP/NIR (**Figure 5**, red) to well D1, 10 µl of secondary conjugate (**Figure 5**, green) to wells D2 to D25, and 15 µl of luminol-peroxide mix to all wells of row E, as indicated in **Figure 6**.

The D-ONE follows this with transfer of the wash buffer (**Figure 5**, blue), biotinylated ladder (**Figure 5**, violet) and samples (**Figure 5**, orange), as described for RePlex chemiluminescence detection, and instructs the operator to centrifuge the plate before proceeding with the automated western blot.

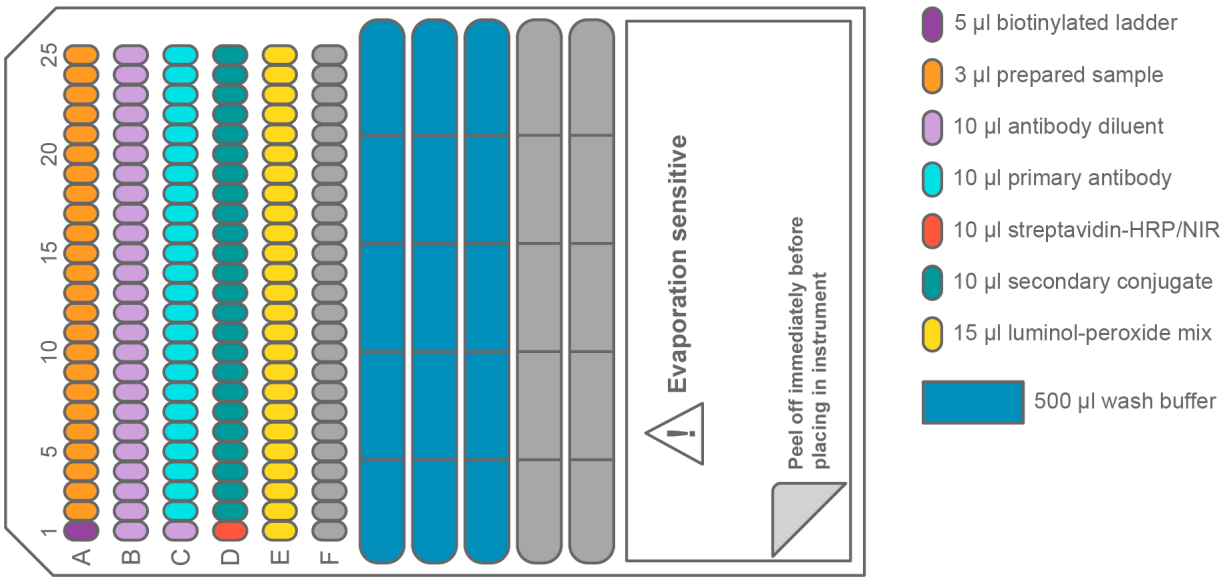

Figure 6: How to fill the Simple Western Jess plate for chemiluminescence analysis.

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Plate set-up for total protein western blot protocol automation

Experimental set-up

Deck Position A: Wash buffer (blue)
Deck Position B: B1 – antibody diluent (lavender), total protein labeling reagent (light green), total protein streptavidin-HRP (green), luminol-peroxide mix (yellow); B3 – biotinylated ladder (violet); B4-B6 – prepared samples (orange, arrow indicates processing direction)

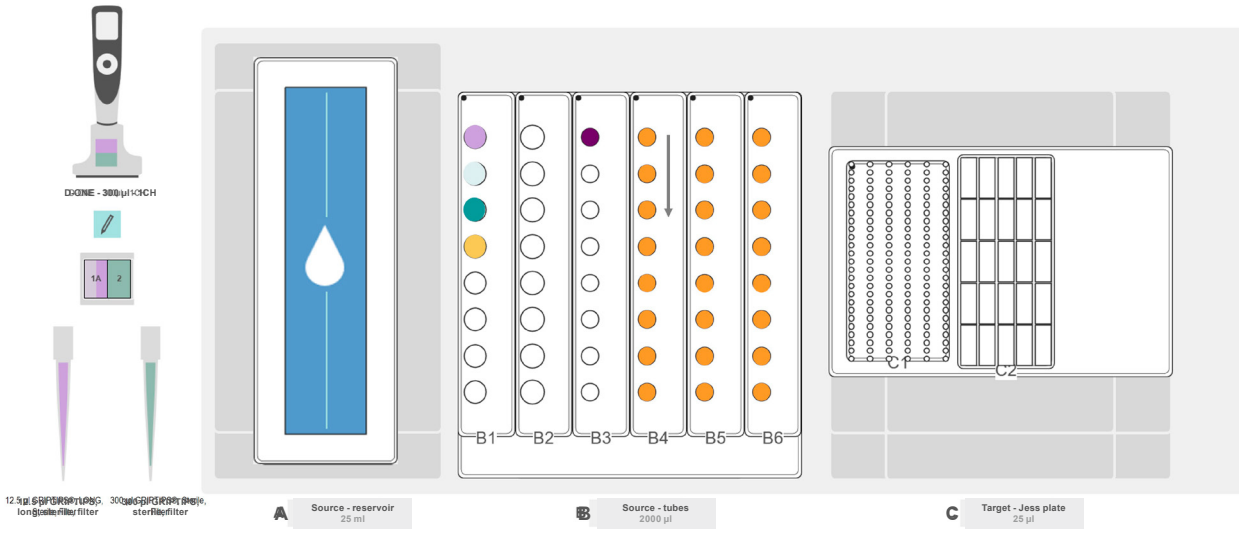

Figure 7: Deck set-up for Jess plate filling to perform total protein analysis. **Position A:** Source – 25 ml reservoir. **Position B:** Source – INTEGRA tube rack for 1.5/2 ml (B1-B2) and 0.5 ml (B3-B6) microcentrifuge tubes. **Position C:** Target – Simple Western Jess plate.

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3. Total protein plate set-up

STEP: Jess plate set-up for total protein analysis.

HOW TO: Set up the ASSIST PLUS deck in a similar way to the chemiluminescence detection protocol, but with the reagents for total protein analysis (**Figure 7**).

Select and run the VIALAB program 'Jess_plate_setup_total_protein'. The D-ONE automatically selects GRIPTIPS, and transfers 10 µl of antibody diluent (**Figure 7**, lavender) into wells B1 and C1 to C25, 8 µl of total protein streptavidin-HRP (**Figure 7**, green) to row D, 15 µl of luminol-peroxide mix (**Figure 7**, yellow) to row E, and wash buffer from the reservoir to the compartment of the Jess plate, as indicated in **Figure 8**. Similar to the western blot protocol for chemiluminescence detection, 5 µl of biotinylated ladder (**Figure 7**, violet) and 3 µl of each sample (**Figure 7**, orange) are also transferred to the Jess plate in Position C. The run is completed by the transfer of 10 µl of total protein labeling reagent (**Figure 7**, light green) into wells B2 to B25, as shown in **Figure 8**. After finishing the liquid transfers, the instrument instructs the operator to centrifuge the plate, as indicated in the kit.

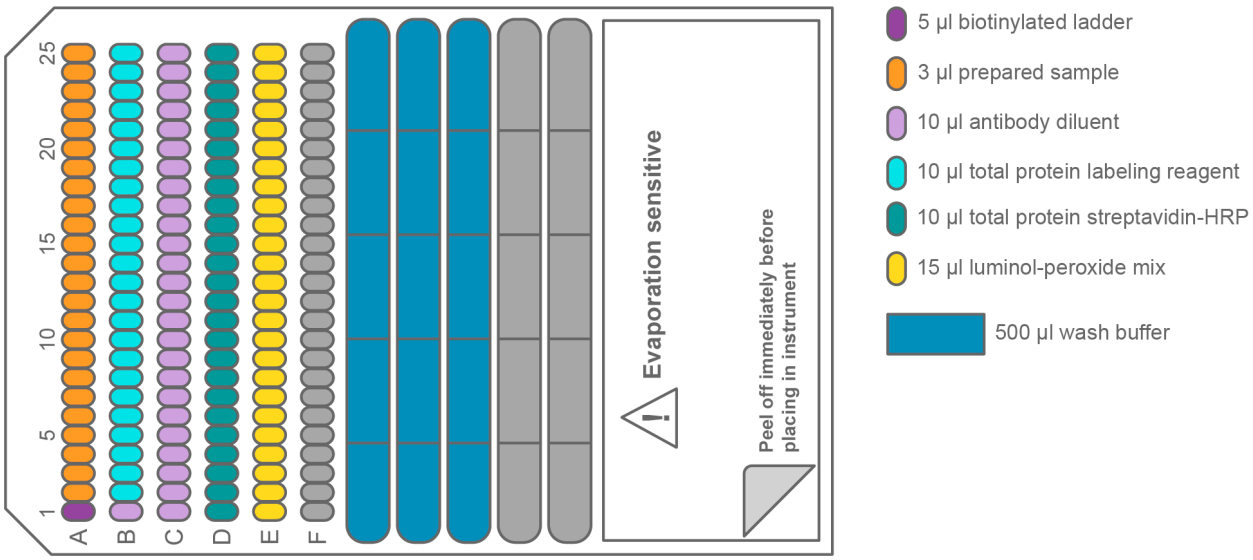

Figure 8: How to fill the Simple Western Jess plate for total protein analysis.

Methods and results

Western blotting can be very time consuming and prone to errors. This application note demonstrates a full, walk-away solution for reliable and high throughput automated western blotting, combining INTEGRA's ASSIST PLUS pipetting robot and D-ONE single channel pipetting module with ProteinSimple's Simple Western Jess.

With RePlex, chemiluminescence and total protein detection were combined in a single assay to prove accurate liquid handling for all reagents when setting up plates with the automated protocols. Reagents, HeLa and C2C12 lysates were prepared according to the protocol in the product insert, with lysate dilutions of 0.64, 0.32, 0.16 and 0.08 mg/ml, together with ready-to-use ERK1 primary and secondary antibodies. Each HeLa or C2C12 dilution was prepared in single tube triplicates as individual samples during plate filling.

Figure 9 shows fully automated western blot normalization of HeLa lysates (lanes 2-13), and C2C12 (lanes 14-25), in triplicate. After ERK1 detection (**Figure 9a**; left), the primary and secondary antibodies were removed with the RePlex reagent mix, to re-stain samples for total protein analysis (**Figure 9a**; right). The data sets generated were automatically analyzed, using the Simple Western software tool to visualize the uncorrected (**Figure 9b**; left) and corrected (**Figure 9b**; right) ERK1 peak target areas. All 3 replicates of each lysate showed great reproducibility while successfully normalizing ERK1 protein levels in 4 different concentrations, proving accurate liquid handling and confirming on-deck reagent/sample stability during plate set-up.

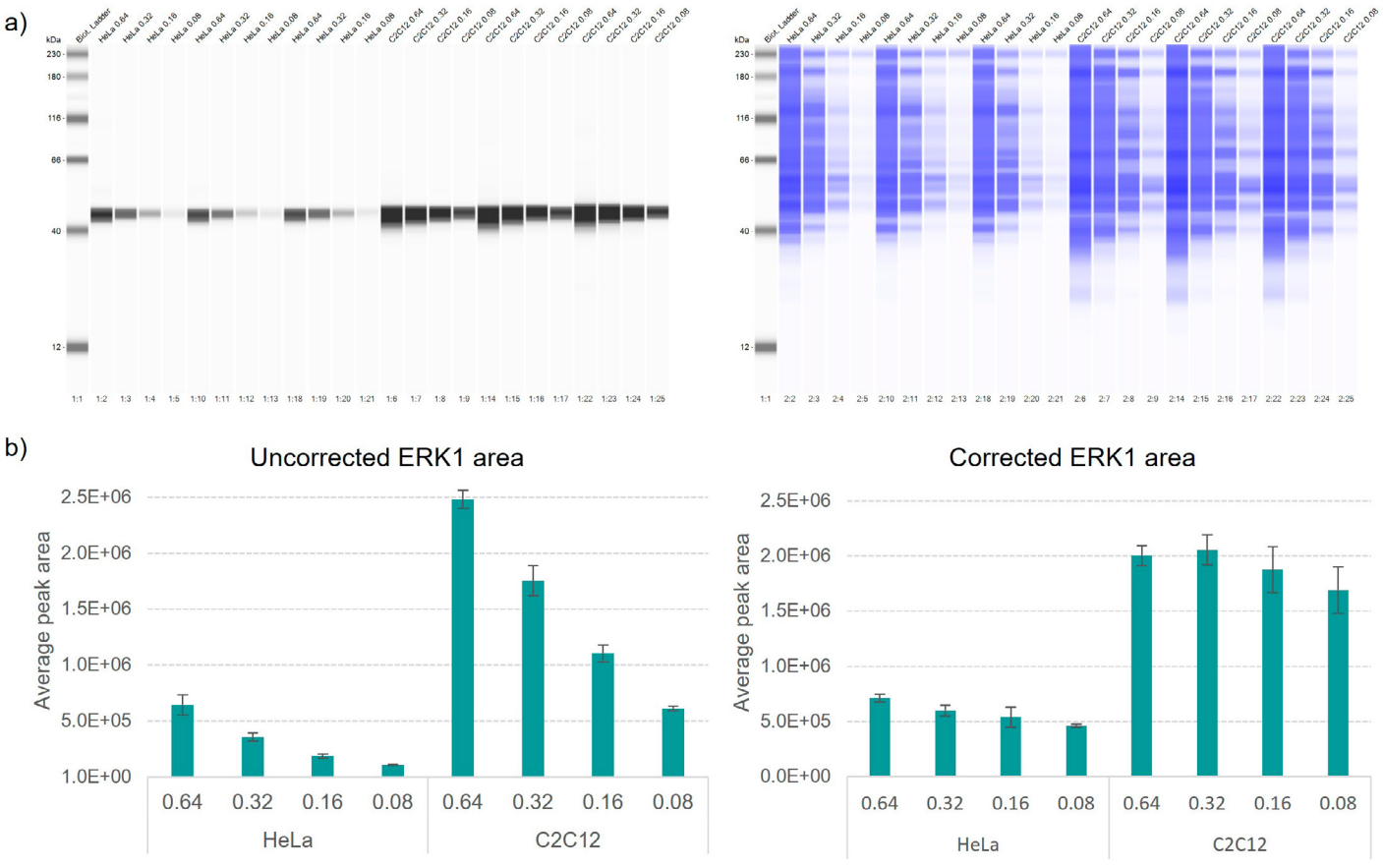

Figure 9: Full walk-away western blot normalization with the ASSIST PLUS, D-ONE and Jess using RePlex. HeLa (lanes 2-13) and C2C12 (lanes 14-25) lysates were each titrated to 0.64, 0.32, 0.16 and 0.08 mg/ml concentrations and run in triplicate.
a) ERK1 detection (left) and total protein detection (right). b) Automatic quantification of uncorrected and corrected ERK1 peak area using the Simple Western software.

Furthermore, simple fluorescence-based detection was carried out to prove the equivalence in performance between automated and manual plate filling, by removing the luminol-peroxide mix from the VIALAB protocol for chemiluminescence detection (Page 6).

Again, reagents and HeLa lysates were prepared according to the protocol in the product insert, with sample dilutions of 0.64, 0.32, 0.16, 0.08, 0.04 and 0.00 mg/ml (0.1x sample buffer). The primary antibody was prepared by diluting 15 µl of HSP60 and 6 µl of ß-actin in 279 µl of milk-free antibody diluent. The secondary antibody was prepared by diluting 15 µl of anti-rabbit IR and 15 µl of anti-mouse NIR in 270 µl of milk-free antibody diluent.

Figure 10a illustrates the results of automated and manual Jess plate set-up (lane view). Wells 1-13 of rows A to D, as well as the wash buffer compartments, were filled using the D-ONE and ASSIST PLUS. Wells 14-25 of rows A to D were filled manually using a single channel pipette. Both filling methods produced reliable fluorescence data when processing the first 3 dilutions of the HeLa lysates in quadruplicate (0.64 and 0.32 mg/ml) and triplicate (0.16 mg/ml), with CVs within the instrument's specifications.

Figure 10b illustrates the results when performing fluorescent detection of all 6 HeLa dilutions, by preparing each dilution in 4 single tube replicates on the ASSIST PLUS deck (lane view and electropherogram showing 0.64 mg/ml replicates as representation). Again, CVs met the instrument specifications while showing high detection sensitivity for low abundance targets when automating plate filling with the D-ONE and ASSIST PLUS.

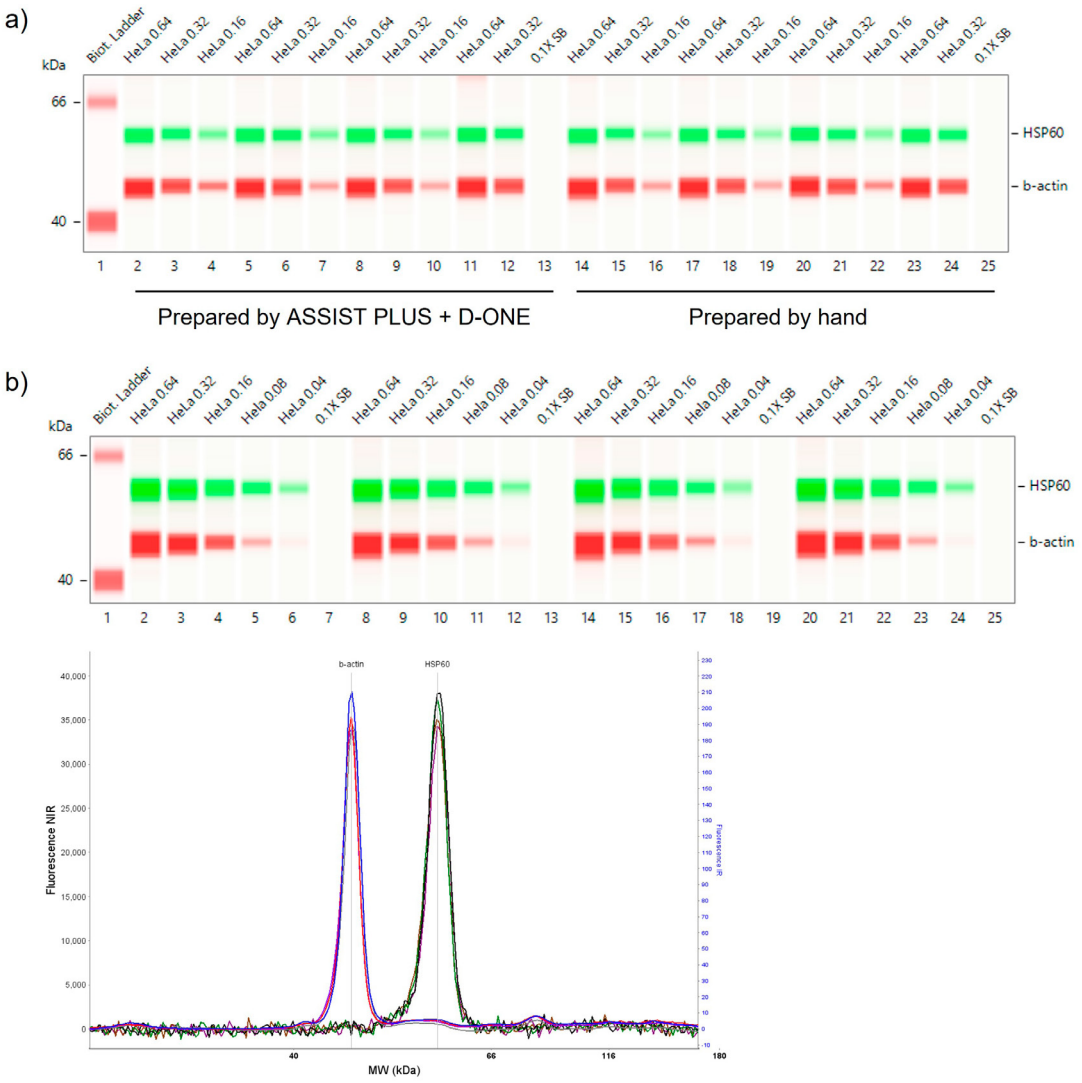

Figure 10: Fluorescence-based detection results demonstrate comparable Jess plate filling. a) Automated (2-13) and manual (14-25) set-up of a Jess plate with various dilutions of HeLa lysates in quadruplicate (0.64 and 0.32 mg/ml) and triplicate (0.16 mg/ml) (lane view). b) Fully automated set-up of 24 samples when processing 4 replicates of 0.64, 0.32, 0.16, 0.08, 0.04 and 0.0 mg/ml (0.1x sample buffer) HeLa lysates (lane view and electropherogram showing 0.64 mg/ml replicates as representation).

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Remarks

- **Labware:** The simple labware creation tool in the VIALAB library makes the integration of special plates easier than ever.
- **VIALAB software:** VIALAB programs can be easily adapted to your specific pipette, labware and protocols.
- **Partial plates:** Programs can be adapted at any time to a different number of samples, giving laboratories total flexibility to meet current and future demands.

Conclusion

- High throughput western blot protocol automation with the ASSIST PLUS pipetting robot, D-ONE single channel pipetting module and Simple Western Jess eliminates user error and demonstrates CVs below instrument specifications.
- The sensitivity of your western blots can be increased with Simple Western advanced capillary electrophoresis and immunodetection technology, and accurate and precise plate set-up can be performed with the D-ONE module for ASSIST PLUS.
- This method can decrease the time needed to run a
- western blot protocol from days to under 3 hours, by using the ASSIST PLUS and D-ONE to automate Simple Western Jess plate filling.
- Western blot workflows can be effortlessly accomplished with RePlex, and total protein normalization performed with ease, or multiplexed with simultaneous chemiluminescent and fluorescent assays on the same sample.

Materials

Manufacturer	Part Number	Description	Link
INTEGRA Biosciences	4505	ASSIST PLUS base unit	https://www.integra-biosciences.com/en/pipetting-robots/assist-plus
INTEGRA Biosciences	4531	D-ONE single channel pipetting module	https://www.integra-biosciences.com/en/pipetting-robots/d-one-for-assist-plus
INTEGRA Biosciences	4535	D-ONE tip deck	https://www.integra-biosciences.com/en/pipetting-robots/d-one-for-assist-plus
INTEGRA Biosciences	4540/4541	Tube rack for 1.5/2.0 ml and 0.5 ml microcentrifuge tubes	https://www.integra-biosciences.com/en/pipetting-robots/assist-plus
INTEGRA Biosciences	4304	25 ml reservoir base	https://www.integra-biosciences.com/en/reagent-reservoirs/multichannel-reagent-reservoirs
INTEGRA Biosciences	4316	25 ml reservoir	https://www.integra-biosciences.com/en/reagent-reservoirs/multichannel-reagent-reservoirs
INTEGRA Biosciences	6405	12.5 µl long, sterile, filter GRIPTIPS	https://www.integra-biosciences.com/en/griptipsr/automation-griptipsr
INTEGRA Biosciences	6435	300 µl standard, sterile, filter GRIPTIPS	https://www.integra-biosciences.com/en/griptipsr/automation-griptipsr
INTEGRA Biosciences	4570	Waste bags	https://www.integra-biosciences.com/en/pipetting-robots/assist-plus
ProteinSimple	004-650	Simple Western Jess System	https://www.bio-techne.com/p/simple-western/jess_004-650
ProteinSimple	042-488	HeLa Lysate Controls	https://www.bio-techne.com/p/simple-western/hela-lysate-controls_042-488
Novus Biologicals (a Bio-Techne brand)	NBP2-10268	C2C12 Whole Cell Lysate	https://www.novusbio.com/products/c2c12-lysate_nbp2-10268
ProteinSimple	042-486	Erk 1 Primary Antibody for Size Assays	https://www.bio-techne.com/p/simple-western/erk-1-primary-antibody-for-size-assays_042-486
Novus Biologicals	MAB8929	Beta-Actin Antibody	https://www.novusbio.com/products/beta-actin-antibody-937215_mab8929
R&D Systems, Inc. (a Bio-Techne brand)	AF1800	HSP60 Antibody	https://www.bio-techne.com/p/antibodies/human-mouse-rat-hsp60-antibody_af1800
ProteinSimple	SM-W004	12-230 kDa Separation Module	https://www.bio-techne.com/p/simple-western/12-230-kda-separation-module_sm-w001
ProteinSimple	SM-FL004	12-230 kDa Fluorescence Separation Module	https://www.bio-techne.com/p/simple-western/12-230kda-fluorescence-separation-module_sm-fl001
ProteinSimple	DM-001	Anti-Rabbit Detection Module	https://www.bio-techne.com/p/simple-western/anti-rabbit-detection-module_dm-001
ProteinSimple	DM-008	Anti-Rabbit IR Detection Module	https://www.bio-techne.com/p/simple-western/anti-rabbit-ir-detection-module_dm-008

ProteinSimple	DM-009	Anti-Mouse NIR Detection Module	https://www.bio-techne.com/p/simple-western/anti-mouse-nir-detection-module_dm-009
ProteinSimple	DM-TP01	Total Protein Detection Module for Chemiluminescence based total protein assays	https://www.bio-techne.com/p/simple-western/total-protein-detection-module-for-chemiluminescence-based-total-protein-assays_dm-tp01
ProteinSimple	RP-001	RePlex Module	https://www.bio-techne.com/p/simple-western/replex-module_rp-001

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