

Human VEGF ELISA Kit

EHVEGF EHVEGF2 EHVEGF5

1165.3

| Number | Description |
|----------------|--|
| EHVEGF | Human VEGF ELISA Kit , sufficient reagents for 96 determinations Kit Contents: Anti-Human VEGF Coated 96-Well Plate , 1 each Lyophilized Recombinant Human VEGF Standard , 2 vials Sample Diluent , 14 ml, contains 0.1% sodium azide 30X Wash Buffer , 50 ml Biotinylated Antibody Reagent , 12 ml, contains 0.1% sodium azide Streptavidin-HRP Reagent , 14 ml TMB Substrate Solution , 13 ml Stop Solution , 13 ml, contains 0.16 M sulfuric acid Adhesive Plate Sealers , 6 each |
| EHVEGF2 | Human VEGF ELISA Kit , sufficient reagents for 2 × 96 determinations Kit Contents: Anti-Human VEGF Coated 96-Well Plate , 2 each Lyophilized Recombinant Human VEGF Standard , 4 vials Sample Diluent , 2 × 14 ml, contains 0.1% sodium azide 30X Wash Buffer , 2 × 50 ml Biotinylated Antibody Reagent , 2 × 12 ml, contains 0.1% sodium azide Streptavidin-HRP Reagent , 2 × 14 ml TMB Substrate Solution , 2 × 13 ml Stop Solution , 2 × 13 ml, contains 0.16 M sulfuric acid Adhesive Plate Sealers , 12 each |
| EHVEGF5 | Human VEGF ELISA Kit , sufficient reagents for 5 × 96 determinations Kit Contents: Anti-Human VEGF Coated 96-Well Plate , 5 each Lyophilized Recombinant Human VEGF Standard , 5 vials Sample Diluent , 75 ml, contains 0.1% sodium azide 30X Wash Buffer , 200 ml Biotinylated Antibody Reagent , 50 ml, contains 0.1% sodium azide Streptavidin-HRP Reagent , 70 ml TMB Substrate Solution , 5 × 13 ml Stop Solution , 55 ml, contains 0.16 M sulfuric acid Adhesive Plate Sealers , 30 each |

For research use only. Not for use in diagnostic procedures.

Storage: For maximum stability, store in a non-defrosting -20°C freezer and refer to the expiration date for frozen storage on the label. Alternatively, store at 2-8°C and refer to the expiration date for refrigerated storage. Once thawed, store at 4°C until the expiration date for refrigerated storage. Kit is shipped on dry ice.



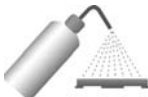





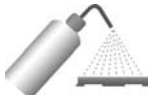



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Introduction

This Thermo Scientific ELISA Kit is for measuring human VEGF in serum; heparin and sodium citrate plasma; and culture supernatants. The microplate provided is coated with anti-human VEGF₁₆₅ antibody that captures VEGF in standards and samples added to the plate. After nonbound proteins are removed, a biotinylated detecting antibody is added and binds to a second site on the VEGF. Excess detecting antibody is removed and streptavidin-horseradish peroxidase is added that reacts with TMB to produce colorimetric signal.

Procedure Summary

- | | | | |
|---|--|--|---|
|  |  |  |  |
| 1. Add 50 µl of Sample Diluent to each well. Add 50 µl of standards and samples. | 2. Cover plate and incubate at room temperature (RT) for 2 hours. | 3. Wash plate THREE times. | 4. Add 100 µl of Biotinylated Antibody Reagent to each well. |
|  |  |  |  |
| 5. Cover plate and incubate at RT for 1 hour. | 6. Wash Plate THREE times. | 7. Add 100 µl of Streptavidin-HRP Reagent to each well. | 8. Cover plate and incubate at RT for 30 minutes. |
|  |  |  |  |
| 9 Wash plates THREE times. | 10. Add 100 µl of TMB Substrate to each well. Develop plate in the dark at RT for 30 minutes. | 11. Stop reaction by adding 100 µl of Stop Solution to each well. | 12. Measure absorbance on a plate reader and calculate results. |

Additional Materials Required

- Precision pipettors with disposable plastic tips to deliver 5-1,000 µl and plastic pipettes to deliver 5-15 ml
- A glass or plastic two-liter container to prepare Wash Buffer
- A squirt wash bottle or an automated 96-well plate washer
- 1.5 ml polypropylene or polyethylene tubes to prepare standards – do not use polystyrene, polycarbonate or glass tubes
- Disposable reagent reservoirs
- 15 ml plastic tube to prepare Streptavidin-HRP Solution
- Standard ELISA reader for measuring absorbance at 450 nm and 550 nm: If a 550 nm filter is not available, the absorbance may be measured at 450 nm only. Refer to the instruction manual supplied with the instrument being used.
- Graph paper or a computerized curve-fitting statistical software package

Precautions

- All samples and reagents must be at room temperature (20-25°C) before use in the assay.
- Review all instructions carefully and verify components against the Kit Contents list (page 1) before beginning the assay.
- Do not use a water bath to thaw samples. Thaw samples at room temperature.
- If using a multichannel pipettor, always use a new disposable reagent reservoir.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Once reagents have been added to the plate, take care NOT to let plate DRY at any time during the assay.
- Avoid microbial contamination of reagents.
- Vigorous plate washing is essential.
- Avoid exposing reagents to excessive heat or light during storage and incubation.
- Do not mix reagents from different kit lots. Discard unused ELISA components after assay completion.
- Do not use glass pipettes to measure TMB Substrate Solution. Take care not to contaminate the solution. If solution is blue before use, DO NOT USE IT.
- Individual components may contain antibiotics and preservatives. Wear gloves while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedures.

Additional Precautions for the 5-plate Kit

- Dispense and equilibrate to room temperature only the reagent volumes required for the number of plates being used. Do not combine leftover reagents with those reserved for additional plates.
- Use only one bottle of the TMB Substrate Solution per 96-well plate. Do not combine leftover substrate with that reserved for other plates.
- Use only one vial of Standard per 96-well plate.

Sample Preparation

Sample Handling

- Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -70°C. Avoid repeat freeze-thaw cycles when storing samples.
- Samples and standards must be assayed in duplicate each time the assay is performed.
- Gradually equilibrate samples to room temperature before beginning assay. Do not use a heated water bath to thaw or warm samples. Mix samples by gently inverting tubes.
- If testing culture supernatants and using a medium other than RPMI with up to 20% FCS, validate the medium to determine compatibility with the assay. To perform this validation, prepare one standard curve using culture medium and another curve using Sample Diluent. If the mean absorbance values for each point on the two curves are within 10% of each other, the medium will not interfere with the assay.
- If samples are clotted, grossly hemolyzed, lipemic or contaminated, or if there is any question about the integrity of a sample, make a note on the template and interpret results with caution.

Sample Dilution

- If the VEGF concentration possibly exceeds the highest point of the standard curve (i.e., 2,000 pg/ml), prepare one or more five-fold dilutions of the sample. When testing **culture supernatants**, prepare dilutions using the culture medium. When testing **serum or plasma**, prepare serial dilutions using the Sample Diluent provided. A five-fold dilution is prepared by adding 50 µl of sample to 200 µl of diluent. Mix thoroughly between dilutions.

Reagent Preparation

For procedural differences when using partial plates, look for **(PP)** throughout these instructions.

Note: When using the 5-plate kit, only one standard per plate is supplied, therefore, partial plates cannot be used.

Wash Buffer

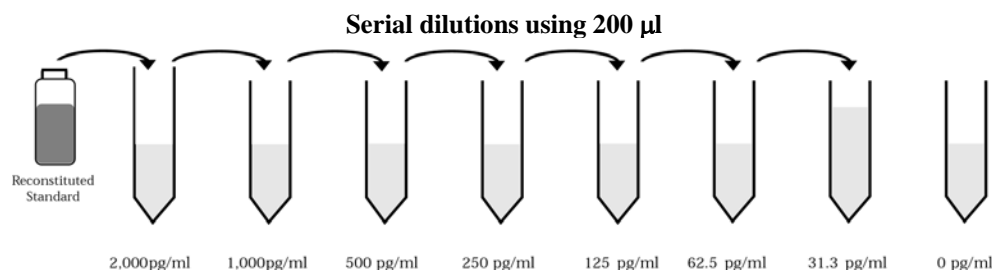
Note: Wash Buffer must be at room temperature before use in the assay. Do not use Wash Buffer if it becomes visibly contaminated during storage.

1. Label a clean glass or plastic two-liter container "Wash Buffer." The 30X Wash Buffer may have a cloudy appearance.
2. If using a 5-plate kit, add 30 ml Wash Buffer to 870 ml water for each plate used, otherwise, add the entire contents of the Wash Buffer (50 ml) bottle to the container. Dilute buffer to 1.5 L with ultrapure water and mix thoroughly.

(PP) When using partial plates, store the reconstituted Wash Buffer at 2-8°C.

Standards

- **(PP)** Reconstitute and use one vial of the lyophilized standard per partial plate.
 - Prepare Standards just before use and use within one hour of reconstitution. Do not store reconstituted standards.
1. Reconstitute standard with ultrapure water. Reconstitution volume is stated on the standard vial label. The standard will dissolve in approximately 1 minute. Mix by gently inverting vial.
 2. Label eight tubes, one for each standard curve point: 2,000; 1,000; 500; 250, 125; 62.5, 31.3 and 0 pg/ml.
 3. Pipette 200 µl of the Sample Diluent into each tube.
 4. Dilute the reconstituted standard 1:2 by adding 200 µl of the reconstituted standard to the tube labeled 2,000 pg/ml, mix thoroughly but gently. Prepare additional 1:2 serial dilutions as follows:
 5. Pipette 200 µl of the 2,000 pg/ml standard into the tube labeled 1,000 pg/ml and mix.
 6. Pipette 200 µl of the 1,000 pg/ml standard into the tube labeled 500 pg/ml and mix.
 7. Perform four additional serial dilutions. Do not add standard to the tube marked 0 pg/ml.



Assay Procedure

A. Sample and Standard Incubation

- Determine number of strips required and leave these strips in the plate frame. Tightly seal remaining unused strips in the provided foil pouch with desiccant and store at 2-8°C. After completing the assay, retain plate frame for second partial plate. When using the second partial plate, place strips securely in the plate frame.
- Use a new pipette tip for each standard or sample transfer.
- Use a new disposable reservoir for each assay reagent.
- Perform all incubations without shaking.
- Use the Data Template provided to record the locations of the human VEGF standards and samples. Assay seven standards and one zero standard in duplicate with each series of unknown samples.

1. Add 50 μ l of Sample Diluent to each well.
2. Add 50 μ l of standard or sample to each well.
3. Cover plate with adhesive plate sealer and incubate for 2 hours at room temperature, 20-25°C.
4. Carefully remove adhesive plate cover. Wash plate as described in the Plate Washing section (section B).

B. Plate Washing

1. Gently squeeze the long sides of plate frame before washing to ensure all strips securely remain in the frame.
2. Empty plate contents. Use a squirt bottle to vigorously fill each well completely with Wash Buffer, then empty plate contents. Repeat procedure two additional times for a total of THREE washes. Blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate all wells and wash THREE times with Wash Buffer, overfilling wells with Wash Buffer. Blot plate onto paper towels or other absorbent material.

C. Biotinylated Antibody and Streptavidin-HRP Incubation

1. Add 100 μ l of Biotinylated Antibody Reagent to each well.
2. Cover plate with adhesive plate sealer and incubate for 1 hour at room temperature, 20-25°C.
3. Thoroughly wash plate THREE times as described in the Plate Washing Section.
4. Add 100 μ l of Streptavidin-HRP Reagent to each well.
5. Cover plate with adhesive plate sealer and incubate for 30 minutes at room temperature, 20-25°C.
6. Thoroughly wash plate THREE times as described in the Plate Washing Section.

D. Substrate Incubation and Stop Step

- Use new disposable reagent reservoirs when adding TMB Substrate and Stop Solutions.
 - Dispense from bottle ONLY the amount of reagent required for the number of wells being used (i.e., 100 μ l per well). Do not use a glass pipette to measure the TMB Substrate.
 - **(PP)** Do not combine leftover substrate with that reserved for the second partial plate. Take care not to contaminate remaining TMB Substrate Solution.
1. Pipette 100 μ l of TMB Substrate Solution into each well.
 2. Allow enzymatic color reaction to develop at room temperature in the dark for 30 minutes. Do not cover plate with aluminum foil or a plate sealer. The reaction yields a blue solution that turns yellow when Stop Solution is added.
 3. After 30 minutes, stop the reaction by adding 100 μ l of Stop Solution to each well.

E. Absorbance Measurement

- Evaluate plate within 30 minutes of stopping the reaction.
- Measure absorbance on an ELISA plate reader set at 450 nm and 550 nm. Subtract 550 nm values from 450 nm values to correct for optical imperfections in the microplate. If 550 nm is not available, measure absorbance at 450 nm only. Omitting the 550 nm measurement will result in higher absorbance values.

F. Calculation of Results

- Generate the standard curve by plotting the average absorbance obtained for each of the standards on the vertical (Y) axis vs. the corresponding human VEGF concentrations on the horizontal (X) axis. Calculate results using a four-parameter logistic curve-fitting software package. If four-parameter is not available, use a point-to-point curve fit.
- Determine the amount of human VEGF in each sample by interpolating from the sample absorbance value using the standard curve.

- If the sample was diluted, multiply the value interpolated by the dilution factor to calculate amount of human VEGF in the sample.
- Absorbance values obtained for duplicates should be within 10% of the mean value. Carefully consider duplicate values that differ from the mean by greater than 10%.

Performance Characteristics

Standard Curve Range: 31.3 to 2,000 pg/ml

Standard Curve Points: 31.3; 62.5; 125; 250; 500; 1,000; 2,000 pg/ml

Calculated Sensitivity: < 8.0 pg/ml

The sensitivity of this assay, or Lower Limit of Detection¹, is the mean signal of zero + 2 standard deviations read in dose from the standard curve. This value is the smallest dose that is not zero with 95% confidence.

Calibration: The standards in this ELISA are calibrated to an in-house human VEGF reference preparation.

Specificity: This ELISA is for measuring natural and recombinant human VEGF₁₆₅. Isoform VEGF₁₂₁ cross-reacts 100% in the assay. Other isoforms were not available to assess cross-reactivity at the time of assay development. Recombinant human VEGF/PlGF heterodimer cross-reacts at ~20% throughout the standard curve range, which is reflective of the VEGF portion of the heterodimer. VEGF Soluble Receptors R1 (Flt-1) and R2 (KDR/Fc chimera) do not exhibit interference at concentrations up to 1,500 pg/ml and 15,000 pg/ml, respectively.

The following cytokines, tested at 1 µg/ml, did not interfere or cross-react in this human VEGF ELISA: human IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12, IL-12p40, IL-13, IL-15, IL-16, IL-17, IL-18, Eotaxin, G-CSF, GM-CSF, GROα, GROβ, IFNα, IFNγ, MCP-1, MCP-2, MCP-3, MCP-4, MIP-1α, MIP-1β, RANTES, TGFβ, TNFβ or VEGF-D; mouse VEGF₁₂₀ or VEGF₁₆₄.

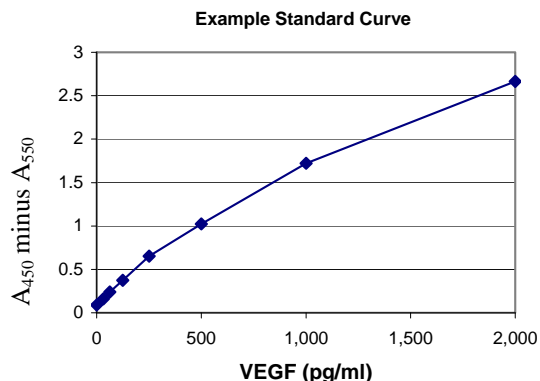
The following substances, tested at 20 µg/ml, did not interfere with this human VEGF ELISA: Concanavalin A (ConA), phytohemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), aprotinin, hemoglobin (HGB), beta-mercaptoethanol (β-ME).

Expected Values: A total of 30 serum and plasma samples collected from apparently healthy individuals were tested in this ELISA. The levels of human VEGF obtained in each sample type are reported below.

| Sample | n | Samples with detectable levels | Mean of detectable samples (pg/ml) | Median (pg/ml) | Range (pg/ml) |
|----------------|----|--------------------------------|------------------------------------|----------------|---------------|
| Serum | 30 | 30 | 182.1 | 196.5 | 22 - 420 |
| EDTA Plasma | 30 | 12 | 59.5 | ND | ND - 86.5 |
| Heparin Plasma | 30 | 15 | 43.4 | ND | ND - 89.0 |
| Citrate Plasma | 30 | 2 | 10.4 | ND | ND - 11.9 |

ND= Not detectable

Precision: Reproducibility was evaluated in each sample matrix. To determine intra-assay precision, 20 replicates of a sample containing low levels of recombinant human VEGF and 20 replicates of a sample containing high levels of recombinant human VEGF were tested on a single plate. To determine inter-assay precision, three different operators tested samples. Each operator performed at least three separate assays on more than one day. Twelve duplicate sample values were used to calculate inter-assay precision data for each level of human VEGF. Data are reported below.



| Sample | Level | Intra-assay Precision | | | Inter-assay Precision | | |
|--------------------------|-------|-----------------------|------------|--------|-----------------------|------------|--------|
| | | Mean (pg/ml) | SD (pg/ml) | CV (%) | Mean (pg/ml) | SD (pg/ml) | CV (%) |
| Serum | 1 | 95.8 | 6.7 | 7.0 | 70.7 | 6.6 | 9.3 |
| | 2 | 1,174 | 56 | 4.8 | 1,224 | 83 | 6.8 |
| EDTA Plasma | 1 | 86.6 | 8.1 | 9.3 | 86.0 | 7.9 | 9.2 |
| | 2 | 1161 | 82 | 7.1 | 1,337 | 80 | 6.0 |
| Heparin Plasma | 1 | 73.8 | 4.8 | 6.4 | 70.2 | 6.3 | 9.0 |
| | 2 | 1,075 | 51 | 4.7 | 1,219 | 119 | 9.7 |
| Citrate Plasma | 1 | 80.2 | 6.2 | 7.8 | 71.8 | 6.8 | 9.5 |
| | 2 | 1021 | 61 | 6.0 | 1,262 | 107 | 8.5 |
| Cell Culture Supernatant | 1 | 72.0 | 6.3 | 8.9 | 74.5 | 7.3 | 9.8 |
| | 2 | 1,147 | 67 | 5.8 | 1,244 | 113 | 9.1 |

Spike and Recovery: Human serum and plasma samples and Sample Diluent controls were spiked with recombinant human VEGF or with natural VEGF from previously assayed synovial fluid. Expected values were calculated by adding endogenous human VEGF levels, from unspiked samples to those of spiked diluent controls. Percent recovery was determined by dividing observed by expected values.

Recombinant Human VEGF

| Sample | Level | n | Expected Value (pg/ml) | Mean Recovery (%) | Median Recovery (%) | Recovery Range (%) |
|----------------|-------|----|------------------------|-------------------|---------------------|--------------------|
| Serum | Low | 8 | 120-163 | 94.0 | 93.6 | 83.4%-102.6 |
| | High | 13 | 454-605 | 103.5 | 104.0 | 90.1%-114.3 |
| EDTA Plasma | Low | 8 | 135-151 | 89.0 | 90.0 | 80.9%-99.3 |
| | High | 13 | 432-559 | 107.0 | 105.7 | 91.9%-119.6 |
| Citrate Plasma | Low | 8 | 136-178 | 91.6 | 91.4 | 82.4%-106.0 |
| | High | 13 | 454-547 | 106.1 | 102.8 | 94.2%-119.6 |
| Heparin Plasma | Low | 8 | 143-214 | 92.0 | 86.3 | 80.4%-114.4 |
| | High | 13 | 465-1,117 | 93.5 | 88.0 | 84.3%-114.7 |

Natural Human VEGF

| Sample | n | Expected Value Range (pg/ml) | Mean Recovery (%) | Median Recovery (%) | Recovery Range (%) |
|--------------------------|----|------------------------------|-------------------|---------------------|--------------------|
| Serum | 11 | 280-699 | 95.8 | 93.6 | 80.5%-120.2 |
| EDTA Plasma | 10 | 165-495 | 94.2 | 94.1 | 85.8%-109.4 |
| Citrate Plasma | 12 | 255-661 | 94.2 | 94.9 | 83.0%-105.4 |
| Heparin Plasma | 10 | 215-636 | 100.5 | 98.9 | 89.7%-111.3 |
| Cell Culture Supernatant | 5 | 270-548 | 87.9 | 88.7 | 80.0%-97.7 |

Linearity of Dilution: Twelve human serum and plasma samples spiked with recombinant or natural human VEGF and six cell culture supernatants spiked with natural human VEGF were serially diluted in Sample Diluent and evaluated in the Human VEGF ELISA. Results for heparin and citrate plasma were similar to those shown for EDTA plasma. Observed values were divided by expected values to calculate percent recovery. Data are reported below.

Recombinant VEGF

| Sample | Dilution | Expected (pg/ml) | Observed (pg/ml) | % Recovery |
|-------------|----------|------------------|------------------|------------|
| Serum | Neat | 496 | --- | --- |
| | 1:2 | 248 | 257 | 104 |
| | 1:4 | 124 | 127 | 102 |
| | 1:8 | 62 | 55 | 87 |
| | 1:16 | 31 | 32 | 102 |
| EDTA Plasma | Neat | 446 | --- | --- |
| | 1:2 | 233 | 222 | 95 |
| | 1:4 | 117 | 120 | 103 |
| | 1:8 | 58 | 55 | 94 |
| | 1:16 | 29 | 32 | 109 |

Natural VEGF

| Sample | Dilution | Expected (pg/ml) | Observed (pg/ml) | % Recovery |
|--------------------------|----------|------------------|------------------|------------|
| Serum | 1:2* | 538 | ---- | ---- |
| | 1:4 | 269 | 259 | 96 |
| | 1:8 | 135 | 148 | 110 |
| | 1:16 | 67 | 72 | 108 |
| EDTA Plasma | 1:2* | 184 | ---- | ---- |
| | 1:4 | 92 | 95 | 103 |
| | 1:8 | 46 | 47 | 102 |
| | 1:16 | 23 | 25 | 109% |
| Cell Culture Supernatant | 1:2* | 222 | ---- | ---- |
| | 1:4 | 111 | 115 | 104 |
| | 1:8 | 56 | 54 | 98 |
| | 1:16 | 28 | 31 | 112% |

*As a result of the viscous properties of synovial fluid used for the natural VEGF spikes, baseline VEGF determinations were made for samples diluted 1:2 with Sample Diluent.

This product ("Product") is warranted to operate or perform substantially in conformance with published Product specifications in effect at the time of sale, as set forth in the Product documentation, specifications and/or accompanying package inserts ("Documentation") and to be free from defects in material and workmanship. Unless otherwise expressly authorized in writing, Products are supplied for research use only. No claim of suitability for use in applications regulated by FDA is made. The warranty provided herein is valid only when used by properly trained individuals. Unless otherwise stated in the Documentation, this warranty is limited to one year from date of shipment when the Product is subjected to normal, proper and intended usage. This warranty does not extend to anyone other than the original purchaser of the Product ("Buyer").

No other warranties, express or implied, are granted, including without limitation, implied warranties of merchantability, fitness for any particular purpose, or non infringement. Buyer's exclusive remedy for non-conforming Products during the warranty period is limited to replacement of or refund for the non-conforming Product(s).

There is no obligation to replace Products as the result of (i) accident, disaster or event of force majeure, (ii) misuse, fault or negligence of or by Buyer, (iii) use of the Products in a manner for which they were not designed, or (iv) improper storage and handling of the Products.

Current versions of product instructions are available at www.thermo.com/pierce. For a faxed copy, call 800-874-3723 or contact your local distributor.

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Data Templates

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |